Influence of Short-Term Maternal Zinc Deficiency on the *In Vitro* Development of Preimplantation Mouse Embryos (43289)

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Abstract. **In this study, we evaluated the use of mouse preimplantation embryos as a model to study zinc deficiency-induced abnormal development. In Experiment 1, the effect of culture medium Zn concentrations on blastocyst development was studied. Preimplantation embryos (2 and 4 cells) obtained from superovulated females developed normally in media containing 0.7–30 μM Zn for up to 72 hr; higher levels of medium Zn resulted in abnormal development. In Experiment 2A, females were fed diets containing 50 (+Zn) or 0.4 (−Zn) μg Zn/g (760 vs 6 nmol/g, respectively) from 1 day before to 1 day after mating (3 days total). Preimplantation embryos were removed from the dams and cultured for 72 hr in 0.7 μM Zn medium. Embryos from the −Zn dams were morphologically normal at time zero; however, over the 72-hr period, these embryos tended to develop at a slower rate than controls, although compaction and cavitation frequency were similar. By the end of the 72-hr culture period, embryos from −Zn dams had significantly fewer cells than did embryos from control dams. In Experiment 2B, an extended period of maternal Zn deprivation (6 days) was used to investigate the potential for further impairment of *in vitro* preimplantation embryo development observed in Experiment 2A. Results from this experiment were consistent with those from Experiment 2A, in addition to providing evidence that the developmental progress of embryos obtained from mice fed Zn-deficient diets for 6 days was significantly impaired. In Experiment 3, the potential for supplemental Zn in culture medium to overcome the impairment in development due to maternal Zn deficiency was investigated. Embryos from female mice subjected to the same dietary regimen described in Experiment 2A were cultured to the blastocyst stage in medium containing Zn at a concentration of either 0.7 or 7.7 μM. Medium Zn supplementation did not improve development of embryos from dams fed Zn-deficient diets. In summary, embryos from mice fed −Zn diets for a 3- or 6-day period encompassing oocyte maturation and fertilization exhibited impaired development *in vitro*. This impairment was not overcome by medium Zn supplementation.** [P.S.E.B.M. 1991, Vol 198]

Severe dietary zinc (Zn) deficiency during pregnancy can have pronounced negative effects on embryonic and fetal development. However, the mechanisms underlying these effects have not been identified. Postulated mechanisms for Zn deficiency-induced abnormalities include: reductions in protein and/or nucleic acid synthesis, abnormal microtubule polymerization, free radical damage, altered gene expression, and altered cell cycles with subsequent distortion in morphogenesis (1). While many of these lesions have been shown to occur in embryos/fetuses obtained from Zn-deficient dams, it is not known which of the events represents direct consequences of embryonic Zn deficiency and which represents secondary phenomena resulting from either an initial embryonic alteration or disturbances in maternal metabolism.

It has been reported that rat embryos removed from dams fed Zn-deficient diets for the first 3 days of gestation were developmentally abnormal compared with controls, as characterized by retarded gestational age and abnormal cell divisions (2). However, it has also been shown that transient exposure to dietary Zn deprivation during the first 6 days of pregnancy, followed by the feeding of an adequate Zn diet, results in term fetuses that are not characterized by gross abnor-

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Received July 2, 1990. [P.S.E.B.M. 1991, Vol 198]
Accepted May 6, 1991.

0037-9727/91/1981-0561$3.00/0

malities (3). Combined, these data suggest that for the rat, there may be substantial embryonic recovery from transient Zn deficiency during preimplantation development. This raises the question of whether the apparent recovery at 6 days is an autonomous direct response of the embryo to Zn in the oviduct, or represents a secondary response of the embryo due to maternal changes associated with the reappearance of adequate dietary Zn.

We are interested in determining whether the mouse preimplantation embryo culture system provides a useful model for investigating the effects of maternal Zn deficiency during early development. Advantages of this model include: the ability to separate maternal from direct factors (with regard to postfertilization events) and control of the environmental Zn concentration of the embryo. In addition, the time period during which the embryos can be studied (2 cells–128 cells) represents a peak period of the ontogeny of numerous gene products and the origins of the two principle stem cell lines of the embryo, inner cell mass, and trophectoderm (4, 5).

Materials and Methods

Mouse Preimplantation Embryo Culture. Random-bred Swiss ICR (CD-1) (Charles River Laboratories, Inc., Shaver Road, MI) female mice fed either stock or semipurified diets (see below) were housed in plastic cages in a temperature- and light-controlled environment (20°C, 14:10-hr light:dark photoperiod). Prior to mating, female mice were superovulated by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG) followed 42–48 hr later by 5 IU of human chorionic gonadotropin (hCG; Sigma Chemical Co., St. Louis, MO). Immediately following superovulation, mice were paired with stock-fed males of the same strain and mated overnight. Forty-eight hr after hCG injection, female mice were killed by cervical dislocation, corpora lutea were counted on individual ovaries, and preimplantation embryos were obtained by flushing the oviducts with a modified Hanks' balanced salt solution (L15; 4) containing 0.5% polyvinylpyrrolidone. At the time they were recovered from oviducts, approximately 80% of embryos had attained the late 2-cell stage of development. Because of the inherent 6- to 12-hr developmental asynchrony exhibited by mouse embryos fertilized *in vivo*, any given cohort of embryos in our experiments exhibited a comparable magnitude of developmental asynchrony. Consequently, many of the 2-cell embryos became 4-cell embryos during the 2 hr required to initiate experiments. Embryos were pooled and divided into groups of 10–30 per culture plate and cultured for 72 hr to the blastocyst stage in a modified (5) bicarbonate-buffered culture medium (T6; 6), in a temperature- and gas-controlled incubator (37°C, 95% O_2 and 5% CO_2). At the end of the culture period, preimplantation embryos were morphologically examined using phase contrast microscopy. Their morphology and developmental stage were considered "normal" if they were comparable to the morphology and developmental stage attained by parallel cultures of embryos from female mice receiving standard care.

After phase contrast microscopic examination for developmental stage, preimplantation embryos were placed in 0.5% sodium citrate for 5 to 15 min, and subsequently fixed onto glass slides using a 1/1 (v/v) acetic acid-absolute ethanol solution and stained for 20 to 30 min with Giemsa stain (7). The number of cells per embryo was obtained by counting stained nuclei using phase contrast microscopy with an eyepiece grid at 50 × magnification.

The following experiments utilized the methods described in the above section. Modifications to this methodology are addressed where appropriate.

Experiment 1: Effect of Medium Zn Concentration on *In Vitro* Development of Preimplantation Mouse Embryos. To determine the effect of varying medium Zn concentration on preimplantation development, embryos were collected from mice fed stock mouse chow (Purina Mouse Chow; Ralston Purina, Stockton, CA: 55 μg Zn/g; 840 nmol/g diet) and cultured for 72 hr in medium containing, by analysis, one of the following concentrations of Zn: 0.7, 3.9, 7.8, 15.4, 30.8, or 60.0 μM. The medium containing 0.7 μM Zn (stock T6) had no added Zn per se; Zn contamination of the reagents used for the media accounted for this concentration of Zn. The remaining concentrations of Zn were achieved by adding 10 μl of concentrated $ZnCl_2$ standards (0.77–12.3 mM) to 1.99 ml of T6 culture medium. Typical plasma and amniotic fluid Zn concentrations in rodents are 15 and 90 μM, respectively (8, 9). Medium Zn concentrations were verified by flame atomic absorbance spectrophotometry (IL 551; Instrumentation Laboratories, Wilmington, MA). Postculture embryos were analyzed for developmental stage and cell number, as described above. Due to limitations in the number of embryos recovered per experiment, not all six concentrations of zinc were tested in the five replicates of this experiment. However, each concentration was tested at least two times and the average number of embryos cultured in respective medium was at least 10. The number of embryos cultured for each group was as follows: control (0.7 μM Zn), $n = 50$; 3.8 μM Zn, $n = 20$; 7.5 μM Zn, $n = 43$; 15.4 μM Zn, $n = 26$; 35 μM Zn, $n = 20$; and 60 μM Zn, $n = 29$. Because there was no significant interexperimental variation in the average cell number among individual groups, the cell numbers obtained for embryos from each group were pooled and a one-way analysis of variance was performed to test for significant differences among experimental groups using Sheffe's test (10). Cell number results are expressed as percent-

age of control embryos, in addition to pooled average cell number.

Experiment 2A: Effect of Acute (3 day) Maternal Zn Deficiency on *In Vitro* Development of Preimplantation Mouse Embryos. To test the idea that maternal Zn deficiency affects embryonic development prior to Day 4 of gestation, female mice were fed either control (50 μg Zn/g, 760 nmol/g) or Zn-deficient diet (0.4 μg Zn/g, 6 nmol/g) for 1 day prior to and 2 days after conception, for a total of 3 days. Initiation of these diets occurred 24 hr after PMSG injection. The detailed composition of the diet has been published (11). Immediately following cervical dislocation, maternal blood samples were obtained by cardiac puncture and pooled due to limited volumes (an average of three per pool), and plasma was obtained following centrifugation at 700*g* for 30 min. Plasma Zn concentration was measured using flame atomic absorbance spectrophotometry.

Embryos obtained from both groups of superovulated mice were cultured to the blastocyst stage in stock T6 medium (0.7 μM Zn) for 72 hr and analyzed as described above. Throughout the culture period, embryos were periodically observed on a temperature-controlled phase microscope stage (37°C) to assess developmental progress. The assessment period never exceeded 4 min per culture dish. Postculture embryos were analyzed for developmental stage and cell number, as described previously. Experiment 2A was conducted on seven separate occasions. A total of 137 embryos from control dams and 164 embryos from dams fed the low Zn diet were assessed for cell number, with at least 10 embryos per group per experiment. Pooled embryo data were analyzed for significance using an unpaired Student's *t* test (10) utilizing the mean of the mean percentage of control embryo cell number.

Experiment 2B: Effect of Acute Maternal Zn Deficiency (6 days) on *In Vitro* Development of Preimplantation Mouse Embryos. Given the results of Experiment 2A, we tested the hypothesis that an extended period of maternal Zn deprivation would further impair *in vitro* development of mouse preimplantation embryos. Female mice were fed either the control or low Zn diet described in Experiment 2A for 4 days prior to and 2 days after conception, for a total of 6 days. Diets were initiated 48 hr before PMSG injection. Maternal blood samples were obtained for plasma Zn analysis, as described in Experiment 2A. Embryos recovered from both groups of mice were cultured for 72 hr, assessed for developmental progress, and analyzed for embryonic cell number, as described previously. Experiment 2B was conducted on three separate occasions. A total of 147 embryos from control dams and 110 embryos from dams fed the low Zn diet were assessed for cell number, with at least 16 embryos per group per experiment. Data are expressed as described in Experiment 2A.

Experiment 3: Effect of Supplemental Medium Zn on Cell Proliferation in Preimplantation Embryos Exposed to Zn Deficiency *In Utero*. To determine the potential for embryonic recovery from Zn deficiency-induced alterations, control embryos and embryos obtained from dams exposed to short-term dietary Zn deprivation were cultured in medium supplemented with Zn. Maternal body weights were measured daily during the 3-day dietary period. Embryos removed from mice subjected to the same dietary and experimental regimes described in Experiment 2 were cultured to the blastocyst stage in medium containing either 0.7 μM Zn (stock T6) or between 7.3 and 8.9 μM Zn (average = 7.7 μM Zn). Maternal blood samples were obtained, pooled, and analyzed for plasma Zn, and corpora lutea were counted, as described previously. Postculture embryos were analyzed as described above. Experiment 3 was replicated four times. The total number of embryos examined for each group was 68, 70, 48, and 66 for the control, low Zn, control + supplemental medium Zn, and low Zn + supplemental medium Zn groups, respectively, with at least 10 embryos per group per experiment. As with Experiments 2A and B, because there was significant interexperimental variation in embryonic cell number ($P < 0.01$), data are expressed as percentage of control embryo (i.e., embryos obtained from control dams cultured on non-supplemented medium) cell number. The mean of the mean cell number per group per experiment was analyzed for significance using one-way analysis of variance and Sheffe's test (10).

Results

Experiment 1: Addition of Zn to the Culture Medium. Between concentrations of 0.7–30.0 μM, Zn added to culture media had no apparent effect on the development of embryos to the expanded blastocyst stage, embryo morphology, or average embryo cell number at the termination of the 72-hr culture period (Table I). In contrast to the above, embryos cultured in the presence of 60 μM frequently failed to cleave, and those that did cleave often exhibited excluded blastomeres and failed to cavitate. In addition, average embryo cell number was significantly lower for embryos cultured in media supplemented with 60 μM Zn compared with controls (embryos cultured in medium containing 0.7 μM Zn).

Experiments 2A (3-day Maternal Zn Deficiency) and 2B (6-day Maternal Zn Deficiency): Development of Cultured Embryos from Dams Fed a Low Zn Diet.

Maternal plasma Zn levels—Experiment 2A. At the time of embryo collection, maternal plasma Zn was significantly lower in mice fed the Zn-deficient diet for 3 days compared with that of mice fed the control diet (10.5 ± 0.9 μM vs 15.6 ± 1.1 μM, $P < 0.01$).

Maternal plasma Zn levels—Experiment 2B. Maternal plasma Zn was significantly lower at the time of

Table I. Influence of Medium Zinc Concentration on Mouse Preimplantation Embryo Cell Number after 72 hr of Culture

Medium Zn concentration (μM)	Percentage of control[a]	n[b]
0.7	100.0 ± 0.0[c]	5
3.8	101.4 ± 16.6	2
7.5	105.2 ± 5.9	4
15.0	95.4 ± 18.3	2
30.0	115.5 ± 12.5	2
60.0	24.7 ± 9.0[d]	2

[a] Values are expressed as means ± SE. Control embryos were defined as those cultured in the 0.7 μM Zn medium.
[b] n = the number of times each concentration was tested with at least 10 embryos.
[c] The mean cell number for control embryos at the end of the 72-hr culture period was 83.3 ± 3.9 (average ± SE).
[d] Significantly lower than the 0.7 μM Zn control group, $P < 0.01$.

Table II. Influence of Maternal Zinc Deprivation on Ovulatory Outcome in Mice

Group	Experiment	Embryo/ corpora lutea[a]	Embryo/ ovary	n[b]
Control	2A	0.86 ± 0.08	11.4 ± 1.7	7
Low Zn	2A	0.94 ± 0.08	14.4 ± 1.8	7
Control	2B	0.95 ± 0.03	12.7 ± 1.3	3
Low Zn	2B	0.82 ± 0.04	9.0 ± 1.3[c]	3

[a] Values expressed as means ± SE.
[b] n = the number of times the experiment was replicated. The average number of embryos per replicate was 20 for Experiment 2A. The average number of embryos per replicate was 17 for Experiment 2B.
[c] Significantly lower than control, $P < 0.04$.

embryo collection in mice fed the low Zn diet for 6 days, compared with that of mice fed the control diet (11.0 ± 3.0 μM vs 20.0 ± 1.0 μM, $P < 0.02$).

Ovulation and embryo yield—Experiment 2A. Based on the number of discernable corpora lutea per ovary, at least 86% of all potential embryos were recovered from oviducts (Table II). The number of corpora lutea per ovary did not differ within or between dietary groups.

There were no significant differences in the numbers of embryos recovered per animal between the dietary groups. Similarly, there were no significant differences in the percentages of 1-cell, 2-cell, or 4-cell embryos, or of degenerating embryos between dietary groups (Table III).

Ovulation and embryo yield—Experiment 2B. At least 82% of all potential embryos were recovered from oviducts of mice that had consumed the experimental diets for 6 days (Table II). The number of corpora lutea per ovary did not differ within or between dietary groups.

In contrast to Experiment 2A, the number of embryos recovered per ovary was significantly lower in mice fed the low Zn diet for 6 days (Table II). Additionally, there was a trend of fewer 1-cell and degenerated embryos, as well as a greater proportion of 2-cell embryos, in the control dams, compared with dams fed the low Zn diet for 6 days (Table III). There were significantly more 4-cell embryos obtained from control mice in Experiment 2B (Table III).

Embryo morphology—Experiment 2A. At the time of their recovery from oviducts, the morphology of embryos obtained from dams fed the low Zn diet (Fig. 1A) was comparable to that of embryos obtained from the control group of dams (Fig. 1B). By the termination of the 72-hr culture period, all embryos obtained from the control mice had developed into expanded blastocysts, some of which had already escaped from their zonae (Fig. 1C). In contrast to the above, the morphology of embryos collected from dams fed the low Zn diet was quite variable after 72 hr of culture. Although some of these embryos (7% ± 3%) experienced cleavage blocks and did not develop into blastocysts, this observation was not statistically significant (Fig. 1D). Embryos from the dams fed the low Zn diets that developed to blastocysts appeared to contain inner cell masses which were smaller than those of blastocysts that developed from embryos obtained from control dams. Nonetheless, some blastocysts in low Zn cultures were escaping from their zonae by the termination of the culture period (Fig. 1D).

Embryo morphology—Experiment 2B. At the initiation of culture, the morphology of recovered embryos obtained from mice fed the low Zn diet was comparable to that of embryos obtained from the control group of dams. By the end of the 72-hr culture period, the morphology of embryos from both groups of dams was consistent with that observed in Experiment 2A.

Developmental kinetics and average embryo cell number—Experiment 2A. The rate of development tended to be delayed in embryos obtained from dams fed the low Zn diet for 3 days, compared with control embryos (Table IV); however, this difference was not statistically significant. This tendency of the embryos from the dams fed the low Zn diet to lag behind their control counterparts was observed throughout the 72-hr period.

In addition to the tendency for a developmental lag, there was a significant reduction in average embryo cell number at the termination of the 72-hr culture period ($P \leq 0.01$). In Experiment 2A, a total of 137 and 164 embryos were examined from the control and Zn-deprived dams, respectively. The average cell number in embryos obtained from control dams at the end of the 72-hr culture period averaged 70, whereas it was 48 in embryos obtained from the Zn-deprived dams. The overall coefficients of variation were 29 and 55% for these groups, respectively. Average embryo cell number at the end of the culture period was consistently lower in the groups of embryos obtained from mice fed

Table III. Influence of Maternal Zinc Deprivation on the Distribution of Mouse Preimplantation Embryos Collected 48 hr after hCG Treatment

Group	Experiment	Embryo stage			Percentage of degenerated embryos	n^b
		1 Cell[a]	2 Cell	>2 Cell		
Control	2A	9.8 ± 6.0	81.3 ± 9.4	5.6 ± 3.9	3.2 ± 3.2	7
Low Zn	2A	6.4 ± 3.9	76.2 ± 6.5	12.5 ± 4.7	4.9 ± 4.9	7
Control	2B	18.2 ± 8.1	40.7 ± 12.5	34.3 ± 8.0	6.8 ± 6.8	3
Low Zn	2B	47.5 ± 18.8	27.2 ± 12.3	7.1 ± 7.1[c]	18.2 ± 9.5	3

[a] Values are expressed as means ± SE. Values represent the percentage in each developmental stage of recovered embryos.
[b] n = the number of times the experiment was replicated. The average number of embryos per replicate was 20 for Experiment 2A. The average number of embryos per replicate was 17 for Experiment 2B.
[c] Significantly lower than control, $P < 0.04$.

low Zn diets compared with controls in all seven experimental replicates.

Developmental kinetics and average embryo cell number—Experiment 2B. Developmental progress of embryos obtained from mice fed the low Zn diet for 6 days was significantly affected, compared with control embryo populations. By Day 2 of culture, there were significantly more degenerating embryos present in the groups obtained from mice fed the low Zn diet for 6 days (Table V). By Day 3 of culture, there were significantly fewer blastocysts, accompanied by a lower frequency of cavitation in the groups of embryos obtained from mice fed the low Zn diet for 6 days (Table V). These differences were consistent with the trends observed in Experiment 2A. It should be pointed out that when control embryo populations that contained a high proportion of 4-cell embryos were excluded from the analysis, these differences were still significant.

In addition to the significant developmental impairment observed with an extended period of maternal Zn deprivation, and consistent with Experiment 2A, average embryo cell number at the end of the 72-hr culture period was significantly lower ($P < 0.01$) in the cultured embryos obtained from mice fed the low Zn diet for 6 days. A total of 147 and 110 embryos were examined from the control and Zn-deprived dams, respectively. The average cell number in embryos obtained from control dams at the end of the 72-hr culture period averaged 78, whereas it was 57 in embryos obtained from Zn-deprived dams. The overall coefficients of variation were 25 and 63% for these groups, respectively. Average embryo cell number at the end of the culture period was consistently lower in the groups of embryos from mice fed the low Zn diet for 6 days compared with controls in all three experimental replicates.

Experiment 3: Adding Zn to the Culture Medium of Embryos from Dams Fed a Low Zn Diet.

Maternal plasma Zn levels. As was the case for Experiments 2A and B, maternal plasma Zn concentrations at the time of embryo collection were significantly lower for low Zn females (11.0 ± 1.3 μM) than for control females (15.7 ± 0.6 μM; $n = 4$ experiments, $P < 0.01$).

Ovulation and embryo yield. Based upon the number of discernable corpora lutea per ovary, 91% of potential embryos were recovered from the oviducts. Similar to results obtained for Experiment 2A, there were no differences in the numbers of corpora lutea per ovary (14 ± 2 vs 14 ± 2 for low Zn and control groups, respectively), or in the initial distribution of embryonic stages recovered per animal between the dietary groups of dams (11 ± 5% vs 10 ± 6% [1-cell embryos], 79 ± 7% vs 79 ± 10% [2-cell embryos], 4 ± 3% vs 0 ± 0% [>2-cell embryos], and 7 ± 5% vs 11 ± 7% [degenerate embryos] for the low Zn and control groups, respectively).

Response of cultured embryos from low Zn dams to Zn added to the culture medium. When culture medium was supplemented with 7.7 μM Zn, the morphology, developmental kinetics, and average embryo cell number at the termination of the 72-hr culture period were all similar to what was observed for the low Zn cultures in Experiments 2A and 2B (data not shown). For the control, low Zn, control + supplemental medium Zn, and low Zn + supplemental medium Zn groups, a total of 68, 70, 48, and 66 embryos were examined for cell number, respectively, with an average of 15 embryos per group per experiment. The average embryo cell numbers for these groups at the end of the culture period were 86 ± 3, 75 ± 3, 80 ± 3, and 74 ± 3 ($n = 4$ experiments, $P < 0.01$), respectively. Average embryo cell number at the end of the culture period was consistently lower in the groups of embryos obtained from mice fed low Zn diets compared with controls in three out of the four experimental replicates. In one replicate, there was no difference among the groups.

Discussion

The results obtained from the current study show that the *in vitro* development of preimplantation mouse embryos is sensitive to maternal Zn deficiency, as evidenced by the impaired developmental progress during culture and the embryonic cell number at the end of the 72-hr culture period. This significantly extends the observations by Hurley and Shrader (2) that preimplan-

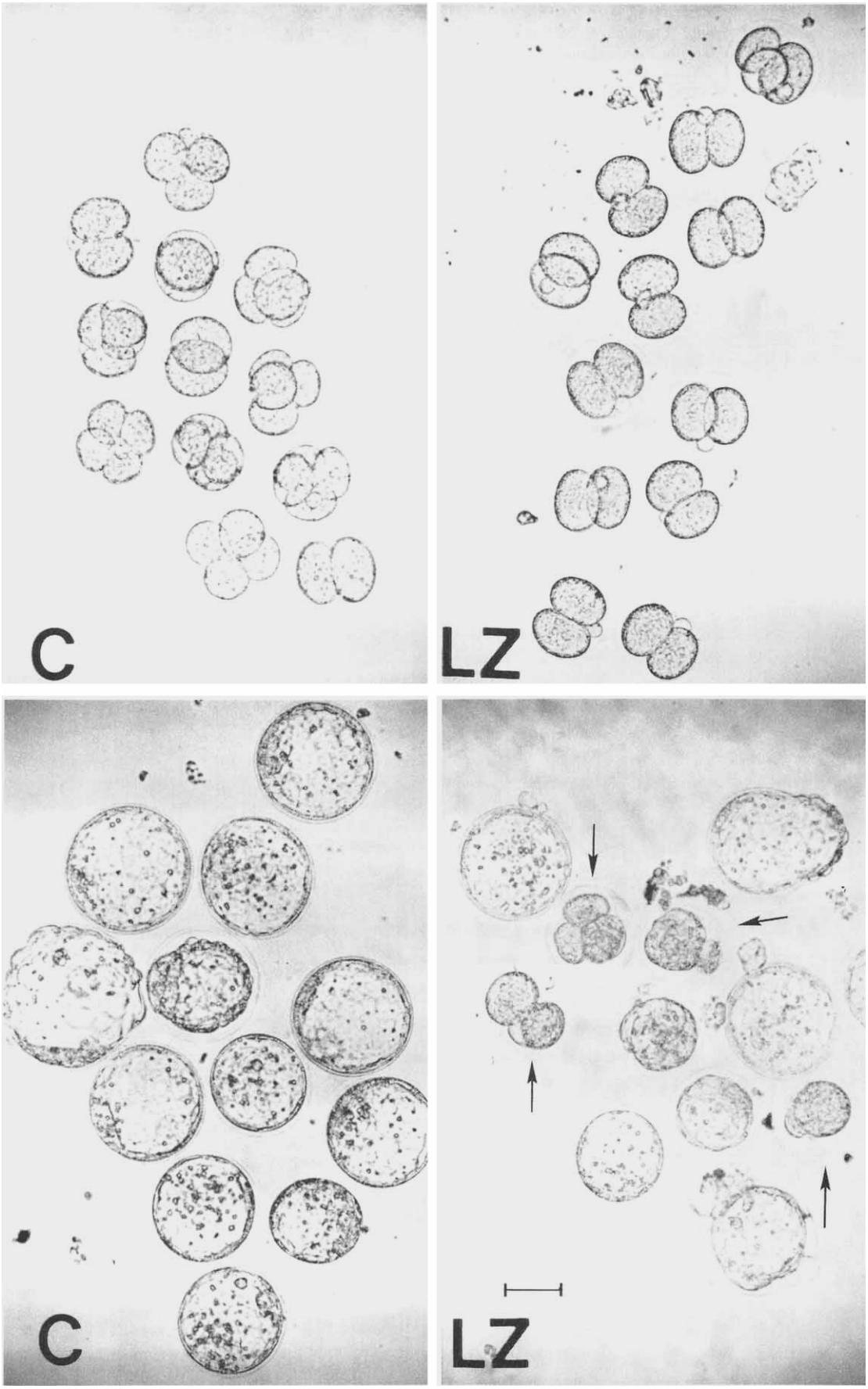

Figure 1. Morphology of mouse embryos at the beginning and end of the culture period. (A) Embryos flushed from the oviducts of control dams. (B) Embryos flushed from the oviducts of Zn-deficient dams. (C) Embryos in Panel A after 72 hr of culture. All the embryos have

Table IV. Kinetic Development of Mouse Embryos *In Vitro* (3 Days of Maternal Zn Deprivation)[a]

Day	Group	Embryo stage				Early morulae ~16 cell embryo	Late morulae ~32 cell embryo	Blastocyst	Degenerate embryo	Percentage undergone cavitation	*n*
		2 cells	4 cells	8 cells	C8[b]						
0	Control	88 ± 7	12 ± 7	—	—	—	—	—	—	—	3
	Low Zn	91 ± 9	9 ± 9	—	—	—	—	—	—	—	3
	P-value	0.4047	0.4047	—	—	—	—	—	—	—	
1	Control	0 ± 0	17 ± 7	16 ± 6	60 ± 10	6 ± 6	—	—	1 ± 1	—	3
	Low Zn	6 ± 3	29 ± 10	16 ± 8	47 ± 6	0 ± 0	—	—	2 ± 2	—	3
	P-value	0.0446	0.1759	0.4861	0.1372	0.1779	—	—	0.2857	—	
2	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	31 ± 15	36 ± 8	31 ± 19	2 ± 1	74 ± 11	3
	Low Zn	1 ± 1	4 ± 4	0 ± 0	1 ± 1	42 ± 16	31 ± 9	15 ± 9	5 ± 2	46 ± 17	3
	P-value	0.1779	0.1779	—	0.1779	0.3199	0.3255	0.2395	0.1245	0.105	
3	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	9 ± 4	89 ± 4	3 ± 1	97 ± 1	3
	Low Zn	1 ± 1	3 ± 3	0 ± 0	0 ± 0	0 ± 0	10 ± 4	79 ± 8	7 ± 3	89 ± 5	3
	P-value	0.1779	0.1779	—	—	—	0.3961	0.1511	0.1211	0.0582	

[a] Cultured embryos were assessed as described in Materials and Methods for developmental progress at indicated time points. Values are expressed as means of percentage of experimental group distribution ± SE. *n* = the number of experimental replicates, with 17 as the average number of embryos per group.
[b] C8 = compacted 8-cell embryo stage.

Table V. Kinetic Development of Mouse Embryos *In Vitro* (6 Days of Maternal Zn Deprivation)[a]

Day	Group	Embryo stage				Early morulae (~16 cell embryo)	Late morulae (~32 cell embryo)	Blastocyst	Degenerate embryo	Percentage undergone cavitation	*n*
		2 cells	4 cells	8 cells	C8[b]						
0	Control	47 ± 2	53 ± 2	—	—	—	—	—	—	—	3
	Low Zn	73 ± 14	28 ± 14	—	—	—	—	—	—	—	3
	P-value	0.0713	0.0713	—	—	—	—	—	—	—	
1	Control	1 ± 1	1 ± 1	3 ± 1	83 ± 5	13 ± 7	—	—	—	—	3
	Low Zn	0 ± 0	4 ± 2	7 ± 2	88 ± 4	1 ± 1	—	—	—	—	3
	P-value	0.1869	0.109	0.1169	0.2418	0.0751	—	—	—	—	
2	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2 ± 1	41 ± 5	56 ± 6	1 ± 1	79 ± 2	3
	Low Zn	0 ± 0	0 ± 0	0 ± 0	1 ± 1	27 ± 25	35 ± 23	32 ± 20	5 ± 1	67 ± 25	3
	P-value	—	—	—	0.1869	0.1873	0.4035	0.1581	0.0078	0.147	
3	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 1	93 ± 2	6 ± 2	96 ± 2	3
	Low Zn	0 ± 0	0 ± 0	0 ± 0	1 ± 1	1 ± 1	7 ± 6	72 ± 9	19 ± 9	79 ± 7	3
	P-value	—	—	—	0.1869	0.1869	0.1612	0.0433	0.1052	0.0406	

[a] Cultured embryos were assessed as described in Materials and Methods for developmental progress at indicated time points. Values are expressed as means of percentage of experimental group distribution ± SE. *n* = the number of experimental replicates, with 17 as the average number of embryos per group.
[b] C8 = compared 8-cell embryo stage.

tation embryos collected from Zn-deficient rats had developed abnormally. The current findings suggest that some of the initial embryonic effects occur prior to the 4-cell stage and that the mouse preimplantation embryo culture system represents a useful model to investigate the direct effects of Zn deprivation during early embryonic development.

Given the observed impairment in early embryonic development induced by transient Zn deficiency, Zn was supplemented to the medium to investigate the potential for embryonic recovery from the maternal Zn deficiency-induced alterations during this period of development. Previous reports indicate that morphologically "normal" term fetuses develop in dams that have been subjected to Zn-deficient diets during early pregnancy followed by adequate Zn feeding (3). However, early preimplantation embryos (2–4 cell) obtained from dams fed Zn-deficient diets failed to overcome the deficit in cell number, despite Zn supplementation of the culture medium. Thus our results suggest that

cavitated, and well-developed inner cell masses are evident in several of them. (D) Embryos in Panel B after 72 hr of culture. Several embryos exhibit abnormal morphology and have failed to cavitate (arrows). In the blastocysts that have developed, inner cell masses are small in comparison with those within control blastocysts. The bar in Panel D represents 100 μm.

preimplantation embryos may not, by themselves, be capable of recovering from maternal Zn deficiency-associated changes, even if supplemental Zn is provided for a prolonged period of time. Possible explanations for this are that the embryonic Zn content affects embryo maturation subsequent to the 4-cell stage, or that supplemental Zn in the culture medium was not available for uptake and/or utilization by the embryo in these experiments. Alternatively, maternal Zn deficiency may affect embryonic development through secondary metabolic changes that are independent of a direct effect of Zn on the embryo.

The optimal medium Zn concentration and the form necessary for preimplantation development is not known. The results from the current study show that mouse preimplantation embryos develop adequately in medium containing Zn at a concentration of 0.7 μM, a value significantly lower than Zn concentrations reported in physiological fluids. To our knowledge, data on mammalian oviduct fluid trace mineral concentrations are not currently available, presumably due to the difficulty in obtaining pure oviduct fluid sample volumes of adequate size to accurately quantify trace mineral concentrations.

The range of culture medium Zn concentrations used in our experiments is similar to ranges commonly used in cell culture systems in which the effects of medium Zn on a variety of parameters, ranging from the protection of preimplantation embryos from cadmium toxicity to the investigations of liver parenchymal cell function, have been assessed (12–15). Our results suggest that a medium Zn concentration of 60 μM is cytotoxic to mouse preimplantation embryos. It is important to point out that the culture medium used in our experiments does not contain serum; thus, the pool of potential Zn binding ligands is presumably low (23 μM bovine serum albumin). This is in contrast to studies in which media are supplemented with serum that would provide additional Zn binding sites (12, 14, 15).

The mechanism(s) underlying the effect of maternal Zn deficiency on preimplantation development cannot be identified based on the results of the current study. Given the time frame of Experiment 2A, the last 24 hr of oocyte maturation, ovulation, oocyte fertilization and/or mitosis, and zygotic gene activation during the early 2-cell stage are all events that might have been altered by the maternal Zn deficiency *in vivo*. When the Zn-deficient diet was introduced at the time of PMSG injection, plasma Zn concentrations in females sacrificed 48 hr after PMSG injection, but prior to hCG, were 16 ± 1 and 10 ± 1 μM Zn ($P \leq 0.01$) in control ($n = 5$) and Zn-deficient ($n = 7$) mice, respectively. In Experiment 2B, the Zn-deficient diet was introduced earlier, 2 days prior to the time of PMSG injection, and plasma Zn concentrations in females sacrificed 48 hr after hCG injection were 11.0 ± 3.0 μM vs 20.0 ± 1.0 μM in deficient and control dams, respectively. Thus, maternal Zn deficiency during the last 24 hr of oocyte maturation in Experiment 2A was accompanied by subsequent impaired embryo development *in vitro* that became more pronounced when maternal Zn deficiency was extended prior to the last 24 hr of oocyte maturation. These observations from mouse preimplantation embryo development *in vitro* support the hypothesis that Zn deficiency affected some aspect of oocyte maturation, which may have been one of the causes for the impaired development *in vivo* by preimplantation embryos recovered from Zn-deficient dams in the rat (2).

This work was supported by NIH Grants HD 01743 (C. L. K.) and HD 16330 (L. M. W.), and National Foundation Grant 15-115 (L. M. W.).

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