Biochemical and Biophysical Research Communications **271,** 250–256 (2000)
doi:10.1006/bbrc.2000.2608, available online at http://www.idealibrary.com on IDEAL®

Zinc-Deficient Rat Embryos Have Increased Caspase 3-like Activity and Apoptosis

Margaret A. Jankowski-Hennig,* Michael S. Clegg,* George P. Daston,† John M. Rogers,‡ and Carl L. Keen*,[1]

**Department of Nutrition, University of California, Davis, California 95616; †Miami Valley Laboratories, Procter and Gamble Company, Cincinnati, Ohio 45239; and ‡Developmental Biology Branch, Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711*

Received March 31, 2000

Caspase activity is a hallmark of apoptosis. Given that maternal zinc (Zn) deficiency results in apoptosis in the rat embryo, we assessed caspase activity in Zn-deficient embryos. Mid-gestation rat embryos were collected from dams fed either a Zn-deficient (0.5 μg Zn/g) diet *ad libitum*, or a Zn-adequate (25 μg Zn/g) diet *ad libitum* or pair fed to dams fed the Zn-deficient diet. Embryos from dams fed the Zn-adequate diet had a normal level of cell death, while embryos from the dams fed the Zn-deficient diet had either increased or normal levels of cell death. Zn-deficient embryos displaying increased cell death had increased caspase activity. Embryos with normal levels of cell death, regardless of maternal diet, had similar caspase activities. Thus, Zn-deficiency-induced apoptosis *in vivo* is associated with increased caspase activity. © 2000 Academic Press

Key Words: **zinc; deficiency; apoptosis; cell death; caspase 3; embryo; development; pregnancy.**

During pregnancy, embryonic and fetal zinc (Zn) deficiency can result in abnormal development of the concepti (1–3). However, despite an awareness of numerous Zn-dependent processes (4–6) the basic mechanisms underlying Zn-deficiency-induced teratogenicity are still poorly understood. We and others have observed increased cell death in rat embryos from dams fed Zn-deficient diets (7–9). This excessive cell death has been characterized as apoptotic, and shown to occur primarily in the optic and head regions, somites, branchial bars and caudal neural crest cells of the embryo (9); structures that are primordia of tissues which often develop abnormally under conditions of Zn deficiency. Based on these observations we have hypothesized that Zn-deficiency-induced cell death in the embryo is one mechanism contributing to Zn-deficiency-induced teratogenicity.

Zn-deficiency-induced cell death in the embryo is related, in part, to maternal food intake patterns and their influence on maternal plasma Zn concentrations (7–9). Rats fed a Zn-deficient diet typically develop a 3-day food intake cycle, in which the intake of food can decrease by more than 50% before returning to control levels. However, the mechanisms underlying the development of this cycle are unknown (10–13). We and others have observed that plasma Zn concentrations are inversely related to the amount of food consumed by the animal (7, 14). Plasma Zn concentrations are lowest following the consumption of control amounts of Zn-deficient diets. When food intake is low, plasma Zn levels are relatively high, presumably due to tissue catabolism and release of sequestered Zn. The above inverse relationship demonstrates that plasma Zn concentrations are not tightly regulated via homeostatic mechanisms. With respect to the developing conceptus, the increased embryonic cell death occurs when maternal plasma Zn concentrations are relatively low. Given that maternal plasma Zn is the primary source of Zn for the developing embryo, it is reasonable to suggest that the low maternal plasma Zn concentrations may trigger the excessive embryonic cell death.

A significant amount of research has focused on the role Zn plays in the apoptotic process (15–17). *In vivo* and *in vitro*, Zn deficiency can result in apoptosis, while Zn supplementation at pharmacological levels can protect cells against a number of diverse apoptotic stimuli (9, 18–35). However, *in vitro* supplementation with excessive levels of Zn can also result in cell death (36–39). Significantly, the concentrations of Zn required in most *in vitro* systems to retard apoptosis are often higher than physiological Zn levels. Zalewski and

[1] To whom correspondence should be addressed at Department of Nutrition, University of California, Davis, One Shields Avenue, Davis, CA 95616. Fax: (530) 752-8966. E-mail: clkeen@ucdavis.edu.

0006-291X/00 $35.00

co-workers have reported that the intracellular concentrations of labile Zn fluctuate during apoptosis, suggesting that modest physiological changes in cellular free Zn concentrations may be a modulator of apoptosis (40, 41). Consistent with this, Duffy *et al.* (1999) demonstrated that cellular concentrations of labile Zn are at their nadir prior to the onset of morphological changes associated with apoptosis (42). Nevertheless, while these observations support the concept that Zn plays a role in the apoptotic process, it is unknown whether cellular fluctuations in Zn concentration are fundamental to the cell death process or if they are just coincidental.

Significant strides have been made in elucidating and describing the biochemical processes and cellular pathways that result in apoptosis. It is now recognized that cysteine aspartate proteases (caspases) are essential to the apoptotic process. Virtually all models of apoptosis are characterized by increased caspase activity. Caspases are a family of proteases that cleave a variety of critical cellular proteins containing the caspase recognition site; DEVD in the case of caspase 3 and 7 (43). Caspase 3, an important mammalian cell death gene (44, 45), can be inhibited *in vitro* by physiologically relevant concentrations of Zn (46, 47). However, whether cellular Zn functions as an inhibitor of caspase 3 activity *in vivo* needs to be further examined.

In this study we tested the hypothesis that Zn-deficient embryos exhibiting increased cell death would have elevated caspase 3-like activity.

MATERIALS AND METHODS

Animals and diet. The protocol used in this study was approved by the Animal Use and Care Administrative Advisory Committee of the University of California at Davis. Virgin Sprague–Dawley female rats (180–200 g; Simonsen Laboratories, Gilroy, CA) were housed individually in stainless steel wire bottom cages, in temperature (22°C) controlled rooms on a normal light cycle (12 h/12 h; lights on 0800/lights off 2000). Upon arrival rats were fed a semi-purified, egg white, Zn-adequate (25 μg Zn/g) diet (control diet). Following at least one week of adaptation, females were mated overnight with males of the same strain. The presence of a sperm plug following the mating period indicated successful breeding and was designated gestation day (GD) 0. On GD 0 dams were assigned to one of three groups: 1) Control (Con); 2) Zn Deficient (ZnD), and 3) Pair Fed (PF). Dams in the control group and the ZnD group were fed their respective diets *ad libitum*. The Zn-deficient ($<$0.5 μg Zn/g) diet is identical to the control diet with the exception that Zn was not added. Dams in the PF group were fed the control diet in quantities equal to those consumed by the dams fed the Zn-deficient diet. In previous studies we have observed that some litters from Zn-deficient dams display levels of increased cell death, while others display levels similar to those of controls (data not shown). Thus, the ZnD group was segregated into two groups on GD 11 based on the level of cell death observed in the embryos. ZnD dams with embryos exhibiting a pattern of increased cell death were assigned to the group ZnDA, while dams with embryos exhibiting a pattern of cell death similar to that of control embryos were assigned to the group designated ZnDB. The PF group was appropriately separated into PFA and PFB groups. Zn concentrations of the diet were verified using inductively coupled plasma spectrophotometry.

Embryo collection. On GD 11 dams were anesthetized with CO_2 and then killed by exsanguination. The embryo and surrounding decidua were explanted from the uterus and placed in Ringer's saline solution (48) warmed to 37°C. Decidua and extraembryonic membranes were removed under a microscope. Embryos were then processed as described below.

Cell death assessment. Two embryos per litter were assessed for pattern and intensity of cell death. Cell death was visualized using the vital stain Nile blue sulfate (49). In preparation for staining, a small transverse incision was made above the forelimbs to facilitate diffusion of the stain into the embryo. Embryos were incubated in Nile blue sulfate staining solution (1:50,000 (w/v) diluted in Ringer's saline solution) for 30 min at 37°C. Embryos were then transferred to fresh Ringer's saline and cell death was evaluated.

Caspase activity analysis. Embryos were cut in half, separating the anterior half (head, heart, and upper trunk) from the posterior half (lower trunk and tail region). Based on developmental stage, anterior portions and posterior portions were grouped in pairs, and then frozen in liquid nitrogen and stored at −80°C. Caspase activity was assessed by adapting the ApoAlert CPP32/Caspase-3 Assay Kit (Clontech, Palo Alto, CA) for tissue analysis. Caspase 3-like activity was determined by monitoring the enzymatic release of a fluorescent compound, 7-amino-4-trifluoromethyl coumarin (AFC), from the caspase specific substrate, DEVD. Briefly, embryonic tissue was sonicated in 100 μl of proprietary lysis buffer, incubated on ice for 10 min and then centrifuged at 12,000 rpm for 3 min at 4°C. The supernate was removed and frozen at −80°C until further processed. A separate aliquot of the same supernate was analyzed for protein concentration using the Bio-Rad *DC* Protein Assay system (Bio-Rad, Hercules, CA). Each aliquot of embryonic supernate assayed for caspase activity was normalized with respect to protein content. Supernates were incubated with the proprietary reaction buffer and substrate at 37°C for 1 h. Sample fluorescence was captured on a scanning fluorometer/platereader (PLS50B; Perkin–Elmer Ltd., Beaconfield, England) using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Caspase 3-like activity in embryonic supernate was confirmed by addition of the caspase 3 specific inhibitor, DEVD-CHO, to the assay reaction prior to the addition of the fluorogenic substrate (50). One sample per litter of posterior sections and two anterior samples per group were assessed.

Zinc analysis. Maternal blood was collected in lithium-heparinized monovette syringes and centrifuged for 15 min at 3000 rpm, 4°C. Maternal plasma and liver were collected, and stored at −20°C until processed. Plasma samples were prepared for analysis by deproteinizing with nitric acid at 4°C for 12 h, and then removing precipitated protein by centrifugation. Liver samples were prepared for analysis as described by Clegg *et al.* (1981) (51). Trace element concentrations were measured using sequential inductively coupled plasma spectrophotometry (Trace Scan; Thermo Jarrell Ash, Wilmington, MA). To ensure accuracy of analysis, banked bovine plasma of known metal content was included as a control. In the case of liver Zn analysis, a National Bureau of Standards bovine liver sample (1577b) served as a control.

Statistical analysis Data were analyzed using one-way ANOVA. Significant differences between groups were analyzed by Fisher's PLSD post hoc tests (52). A P-value $\leq$0.05 was considered significantly different.

RESULTS

Maternal Parameters

Food intake. Dams in the control group consumed a relatively constant amount of diet each day (Fig. 1). In contrast, the food consumption of rats in the Zn-deficient groups varied significantly on a daily basis.

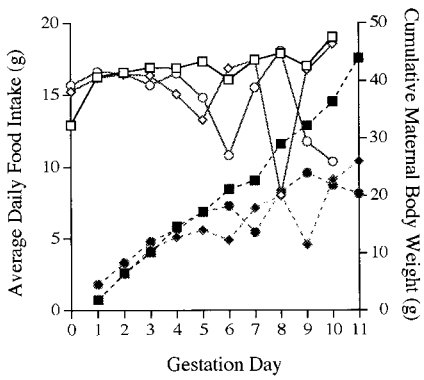

FIG. 1. Maternal average daily food intake and maternal cumulative body weight gain. Maternal average food intake: Control (open square), ZnDA (open diamond), and ZnDB (open circle). Maternal cumulative weight gain: Control (filled square), ZnDA (filled diamond), and ZnDB (filled circle).

Consistent with previous observations by our group, the different feeding patterns observed in the Zn-deficient group were associated with differences in the amount and pattern of cell death in the embryo. Dams in the ZnDA group were characterized by a reduction in food intake first occurring on GD 4, control levels of consumption on GD 7, 9, and 10, and lower quantities on GD 8 (Fig. 1). In contrast, ZnDB dams first began cycling their food intake on GD 5, consumed control amounts of food on GD 7 and 8, and lower amounts on GD 9 and 10.

Weight gain. Dams in all groups had a similar body weight on GD 0 (203.7 ± 1.9 g). Control dams exhibited a linear cumulative weight gain of approximately 4 g/day throughout gestation (Fig. 1). In contrast, ZnDA and ZnDB exhibited variable gains that reflected their respective food intake pattern. By GD 8, maternal cumulative body gain in both Zn-deficient groups was significantly lower than in controls and remained so for the remainder of the experiment. Dams in the PF groups had similar weight gains as dams in the ZnD groups, except on GD 4 and 5 when weight gains of dams in the ZnDA group were statistically higher than in the PFA group.

Tissue Zn concentrations. ZnDA and ZnDB group dams had similar liver Zn concentrations that were lower than all other groups (Table 1). Dams fed Zn-deficient diet had lower plasma Zn concentrations relative to the Con and PF groups. With respect to the ZnD groups, ZnDA dams had lower plasma Zn concentrations than the ZnDB group on GD 11 (Table 1). The hierarchy with respect to plasma Zn concentrations can be summarized as follows: ZnDA < ZnDB < PFA = PFB = Con.

Embryo Cell Death and Caspase Activity

Figure 2a shows the typical pattern and intensity of normal cell death occurring at this developmental stage, consisting of cell death in the optic, otic, olfactory, and dorsal most region of the anterior neural tube, the branchial bars and the first few anterior somites. ZnDB and PF group embryos exhibited a similar pattern and amount of cell death as control embryos. In contrast, ZnDA embryos had increased cell death in the optic, otic, and the dorsal most region of the posterior neural tube, the branchial bars, somites and forelimb buds (Fig. 2b).

In agreement with the level of cell death and maternal plasma Zn concentrations, ZnDA group embryos had higher caspase activity which was associated with both the posterior and the anterior sections (Figs. 3a and 3b). Caspase activity in the posterior sections of the ZnDB group embryos was similar to the PF and control groups. Given that PF groups had similar caspase activity as control groups in the posterior embryo sections, PF groups were not further assessed. ZnDB group embryos had similar caspase activity in the anterior sections as control embryos.

DISCUSSION

Our group has proposed that one mechanism underlying Zn-deficiency-induced teratogenesis is abnormal cell death. While exogenous Zn can inhibit morphological and biochemical indices of apoptosis in cell culture systems, the mechanisms underlying this protection are poorly understood. The purpose of this study was to determine if elevated caspase 3-like activity was associated with the increased cell death in Zn-deficient embryos.

Consistent with our previous work (8, 9), Zn-deficiency-induced cell death was evident in GD 11 rat embryos. Embryos from Zn-deficient dams had either increased cell death or a normal pattern and intensity of cell death. ZnD group embryos characterized by having "normal" cell death levels had caspase 3-like activities that were similar to those of control embryos. In contrast, ZnD group embryos with increased cell death had elevated caspase 3-like activity. Thus, similar to *in vitro* conditions of Zn-deficiency-induced cell death, as

TABLE 1

The Influence of Maternal Zn Deficiency on Maternal Plasma and Liver Zn Concentrations

	N^a	Plasma[b] (μM)	Liver[b] (μmol/g)
Control	6	16.3 ± 0.3*	0.51 ± 0.01*
PFA	4	16.4 ± 0.8*	0.48 ± 0.01*
PFB	4	16.0 ± 0.8*	0.59 ± 0.05❖
ZnDA	7	2.6 ± 0.2†	0.32 ± 0.01†
ZnDB	6	6.6 ± 1.1❖	0.34 ± 0.01†

[a] N, the number of litters in each group.
[b] Values with different superscript symbols, within a column, are statistically different at $P \leq 0.05$.

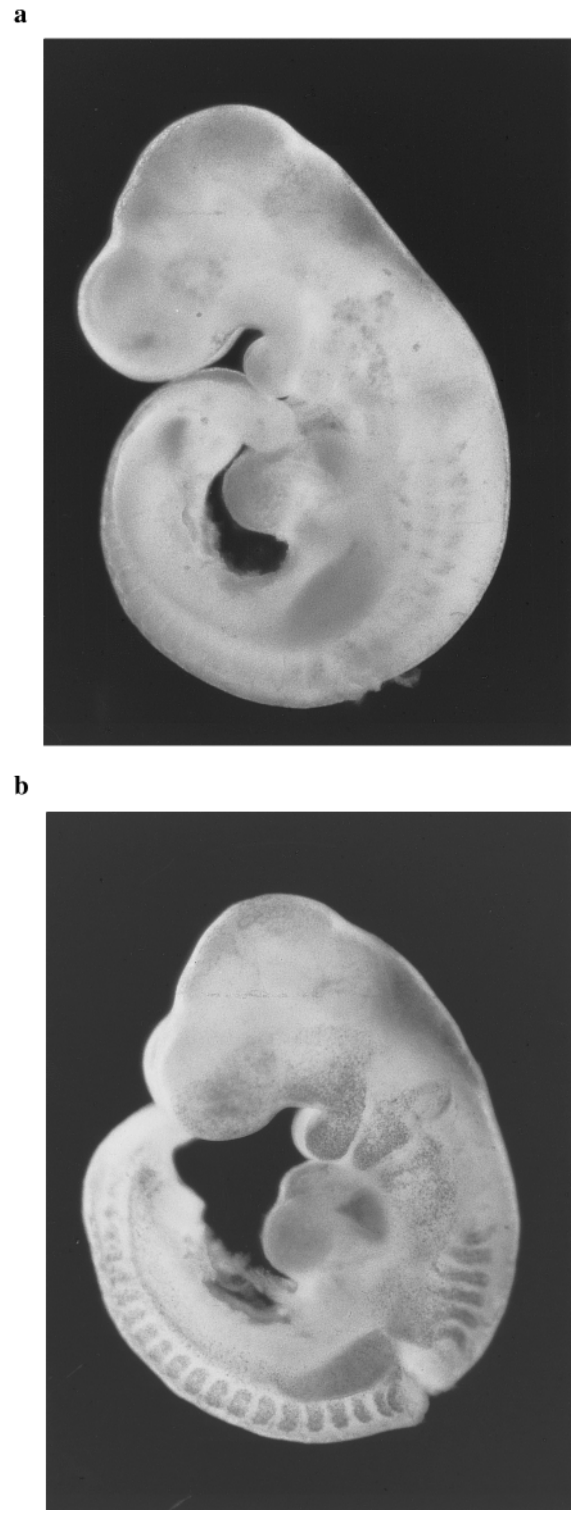

FIG. 2. Cell death patterns in GD 11 rat embryos. The typical pattern and intensity of cell death in a control embryo (a), and the typical pattern and intensity of increased cell death in a ZnDA embryo (b).

well as other cellular models of apoptosis, *in vivo* Zn deficiency in the embryo is associated with elevated caspase 3-like activity.

Increased cell death and high caspase 3-like activity in Zn-deficient embryos was associated with low maternal plasma Zn concentrations. Given that maternal plasma Zn provides Zn for the developing embryo (14, 53), we suggest that the resultant low concentrations of Zn in the embryo (54) contribute to the excessive cell death that is observed. In other animal models, Zn-deficiency-induced cell death occurs in cells undergoing a high rate of cell proliferation—an event requiring many Zn-dependent processes (18, 55). Based upon these studies, it has been suggested that Zn-deficiency-induced cell death results when the available Zn cannot meet the cell's requirement for Zn. Given that the increased cell death in the Zn-deficient embryo is not equally distributed, but occurs in specific areas, we suggest that the occurrence of cell death in Zn-deficient embryos occurs in areas of cell proliferation or where Zn demands are particularly high.

Recently, investigators have shown that physiological levels of Zn can inhibit caspase 3 activity in cell-free systems and procaspase 3 processing into its active form (46, 47, 56, 57). Thus, it is possible that intracellular Zn may be involved in the regulation of *in vivo* caspase 3-like protease activity. In addition, Zn may inhibit constitutively activated caspase 3-like pro-

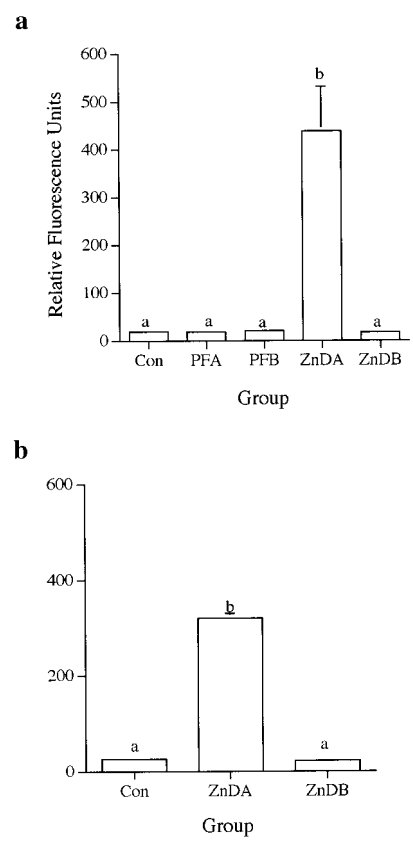

FIG. 3. Embryo caspase activity. Caspase activity present in the posterior portion of the embryo (a). Caspase activity present in the anterior portion of the embryo (b).

teases in non-apoptotic cells. Given the above, it is reasonable to assume that when cellular Zn concentrations decrease below a "threshold" level, Zn inhibition is relieved, and then Zn-sensitive caspases can cleave essential proteins which culminate in cell death.

Inadequate cellular Zn could also cause apoptosis indirectly. Investigators have shown that in animal and cell culture models, Zn deficiency is associated with increased levels of reactive oxygen species (ROS) and oxidative damage (58–61). Given that excessive ROS are proposed as one stimulus initiating apoptosis (32, 62), Zn-deficiency-induced apoptosis may be mediated via increased ROS concentrations. We have observed increased concentrations of ROS and apoptosis in 3T3 cells cultured in Zn-deficient medium (57, 63). Moreover, Carmody *et al.* (1999) have shown that Zn supplementation, albeit at pharmacological levels, can inhibit ROS generation and apoptosis in primary cultures of retinal cells (32). Ethanol-induced teratogenesis is also proposed to be mediated via increased ROS species, and similar to Zn deficiency, results in increased cell death in the embryo. Cartwright *et al.* (1998) demonstrated that ethanol-induced cell death in the embryo is characterized by caspase 3-like activity, while Chen *et al.* (1996) have shown that antioxidants can prevent ethanol-induced death of embryo cells (64, 65). These observations support the concept that ROS can mediate cell death occurring *in vivo,* and thus a similar phenomenon may occur in Zn-deficient embryos.

Finally, glucocorticoids are also a potent stimulant of apoptosis in various cell types, particularly immune cells. Significantly, Fraker and co-workers have observed that Zn deficiency in non-pregnant mice results in increased concentrations of circulating glucocorticoids and in thymocyte apoptosis (66, 67). Moreover, by demonstrating that adrenalectomy can prevent Zn-deficiency-induced thymocyte apoptosis, Fraker *et al.* (1995) have provided strong evidence that the elevation of *in vivo* glucocorticoids can mediate apoptosis associated with Zn deficiency (68). Whether or not an increase in glucocorticoids impacts all cell types and/or contributes to the increased cell death occurring in Zn-deficient embryos is unknown. In addition, Telford *et al.* (1997) have shown that Zn can affect the affinity of glucocorticoids for their receptors. Thus, lower Zn concentrations may effectively result in more glucocorticoid binding to its receptor (69). Thus this may be a second way in which Zn-deficiency-induced alterations in glucocorticoid metabolism may cause cell death in the embryo.

While the potential mechanisms underlying Zn-deficiency-induced cell death in the embryo are numerous, we have begun to identify biochemical characteristics of the dying cell. With this information we can begin to determine the endogenous pathways involved in Zn-deficiency-induced apoptosis in the embryo which will lead to the identification of potential apoptotic stimuli.

ACKNOWLEDGMENTS

The authors thank Amie Hwang for technical assistance. This work was supported by National Institutes of Health Grants HD-01743 and DK-35747.

REFERENCES

1. Peters, J. M., Wiley, L. M., Zidenberg-Cherr, S., and Keen, C. L. (1991) Influence of short-term maternal zinc deficiency on the *in vitro* development of preimplantation mouse embryos. *Proc. Soc. Exp. Biol. Med.* **198,** 561–568.
2. Hurley, L. S., Gowan, J., and Swenerton, H. (1971) Teratogenic effects of short-term and transitory zinc deficiency in rats. *Teratology* **4,** 199–204.
3. Rogers, J. M., Keen, C. L., and Hurley, L. S. (1985) Zinc deficiency in pregnant Long-Evans hooded rats: Teratogenicity and tissue trace elements. *Teratology* **31,** 89–100.
4. Vallee, B. L., and Falchuk, K. H. (1993) The biochemical basis of zinc physiology. *Physiol. Rev.* **73,** 79–118.
5. Bettger, W. J., and O'Dell, B. L. (1981) A critical physiological role of zinc in the structure and function of biomembranes. *Life Sci.* **28,** 1425–1438.
6. Clegg, M. S., Keen, C. L., and Hurley, L. S. (1989) Biochemical pathologies of zinc deficiency. *In* Zinc in Human Biology (Mills, C. F., Ed.), pp. 129–145.
7. Record, I. R., Dreosti, I. E., Tulsi, R. S., and Manuel, S. J. (1986) Maternal metabolism and teratogenesis in zinc-deficient rats. *Teratology* **33,** 311–317.
8. Jankowski, M. A., Uriu-Hare, J. Y., Rucker, R. B., Rogers, J. M., and Keen, C. L. (1995) Maternal zinc deficiency, but not copper deficiency or diabetes, results in increased embryonic cell death in the rat: Implications for mechanisms underlying abnormal development. *Teratology* **51,** 85–93.
9. Rogers, J. M., Taubeneck, M. W., Daston, G. P., Sulik, K. K., Zucker, R. M., Elstein, K. H., Jankowski, M. A., and Keen, C. L. (1995) Zinc deficiency causes apoptosis but not cell cycle alterations in organogenesis-stage rat embryos: effect of varying duration of deficiency. *Teratology* **52,** 149–159.
10. Browning, J. D., MacDonald, R. S., Thornton, W. H., and O'Dell, B. L. (1998) Reduced food intake in zinc deficient rats is normalized by megestrol acetate but not by insulin-like growth factor-I. *J. Nutr.* **128,** 136–142.
11. Kennedy, K. J., Rains, T. M., and Shay, N. F. (1998) Zinc deficiency changes preferred macronutrient intake in subpopulations of Sprague–Dawley outbred rats and reduces hepatic pyruvate kinase gene expression. *J. Nutr.* **128,** 43–49.
12. Lee, R. G., Rains, T. M., Tovar-Palacio, C., Beverly, J. L., and Shay, N. F. (1998) Zinc deficiency increases hypothalamic neuropeptide Y and neuropeptide Y mRNA levels and does not block neuropeptide Y-induced feeding in rats. *J. Nutr.* **128,** 1218–1223.
13. Selvais, P. L., Labuche, C., Nguyen, X. N., Ketelslegers, J. M., Denef, J. F., and Maiter, D. M. (1997) Cyclic feeding behaviour and changes in hypothalamic galanin and neuropeptide Y gene expression induced by zinc deficiency in the rat. *J. Neuroendocrinol.* **9,** 55–62.
14. Masters, D. G., Keen, C. L., Lonnerdal, B., and Hurley, L. S. (1983) Zinc deficiency teratogenicity: The protective role of maternal tissue catabolism. *J. Nutr.* **113,** 905–912.

15. Sunderman, F. W., Jr. (1995) The influence of zinc on apoptosis. *Ann. Clin. Lab. Sci.* **25,** 134–142.
16. Fraker, P. J., and Telford, W. G. (1997) A reappraisal of the role of zinc in life and death decisions of cells. *Proc. Soc. Exp. Biol. Med.* **215,** 229–236.
17. Chai, F., Truong-Tran, A. Q., Ho, L. H., and Zalewski, P. D. (1999) Regulation of caspase activation and apoptosis by cellular zinc fluxes and zinc deprivation: A review. *Immunol. Cell Biol.* **77,** 272–278.
18. Elmes, M. E. (1977) Apoptosis in the small intestine of zinc-deficient and fasted rats. *J. Pathol.* **123,** 219–223.
19. Osati-Ashtiani, F., King, L. E., and Fraker, P. J. (1998) Variance in the resistance of murine early bone marrow B cells to a deficiency in zinc. *Immunology* **94,** 94–100.
20. Thomas, D. J., and Caffrey, T. C. (1991) Lipopolysaccharide induces double-stranded DNA fragmentation in mouse thymus: Protective effect of zinc pretreatment. *Toxicology* **68,** 327–337.
21. Matsushita, K., Kitagawa, K., Matsuyama, T., Ohtsuki, T., Taguchi, A., Mandai, K., Mabuchi, T., Yagita, Y., Yanagihara, T., and Matsumoto, M. (1996) Effect of systemic zinc administration on delayed neuronal death in the gerbil hippocampus. *Brain Res.* **743,** 362–365.
22. Martin, S. J., Mazdai, G., Strain, J. J., Cotter, T. G., and Hannigan, B. M. (1991) Programmed cell death (apoptosis) in lymphoid and myeloid cell lines during zinc deficiency. *Clin. Exp. Immunol.* **83,** 338–343.
23. Treves, S., Trentini, P. L., Ascanelli, M., Bucci, G., and Di Virgilio, F. (1994) Apoptosis is dependent on intracellular zinc and independent of intracellular calcium in lymphocytes. *Exp. Cell Res.* **211,** 339–343.
24. McCabe, M. J., Jr., Jiang, S. A., and Orrenius, S. (1993) Chelation of intracellular zinc triggers apoptosis in mature thymocytes. *Lab. Invest.* **69,** 101–110.
25. Jiang, S., Chow, S. C., McCabe, M. J., Jr., and Orrenius, S. (1995) Lack of Ca^{2+} involvement in thymocyte apoptosis induced by chelation of intracellular Zn^{2+}. *Lab. Invest.* **73,** 111–117.
26. Ahn, Y. H., Kim, Y. H., Hong, S. H., and Koh, J. Y. (1998) Depletion of intracellular zinc induces protein synthesis-dependent neuronal apoptosis in mouse cortical culture. *Exp. Neurol.* **154,** 47–56.
27. Sakabe, I., Paul, S., Dansithong, W., and Shinozawa, T. (1998) Induction of apoptosis in Neuro-2A cells by Zn^{2+} chelating. *Cell Struct. Funct.* **23,** 95–99.
28. Fukamachi, Y., Karasaki, Y., Sugiura, T., Itoh, H., Abe, T., Yamamura, K., and Higashi, K. (1998) Zinc suppresses apoptosis of U937 cells induced by hydrogen peroxide through an increase of the Bcl-2/Bax ratio. *Biochem. Biophys. Res. Commun.* **246,** 364–369.
29. Provinciali, M., Di Stefano, G., and Fabris, N. (1995) Dose-dependent opposite effect of zinc on apoptosis in mouse thymocytes. *Int. J. Immunopharmacol.* **17,** 735–744.
30. Mathieu, J., Ferlat, S., Ballester, B., Platel, S., Herodin, F., Chancerelle, Y., Mestries, J. C., and Kergonou, J. F. (1996) Radiation-induced apoptosis in thymocytes: Inhibition by diethyldithiocarbamate and zinc. *Radiat. Res.* **146,** 652–659.
31. Parat, M. O., Richard, M. J., Pollet, S., Hadjur, C., Favier, A., and Beani, J. C. (1997) Zinc and DNA fragmentation in keratinocyte apoptosis: Its inhibitory effect in UVB irradiated cells. *J. Photochem. Photobiol. B* **37,** 101–106.
32. Carmody, R. J., McGowan, A. J., and Cotter, T. G. (1999) Reactive oxygen species as mediators of photoreceptor apoptosis *in vitro*. *Exp. Cell Res.* **248,** 520–530.
33. Adler, M., Shafer, H., Hamilton, T., and Petrali, J. P. (1999) Cytotoxic actions of the heavy metal chelator TPEN on NG108-15 neuroblastoma-glioma cells. *Neurotoxicology* **20,** 571–582.
34. Ishido, M., Suzuki, T., Adachi, T., and Kunimoto, M. (1999) Zinc stimulates DNA synthesis during its antiapoptotic action independently with increments of an antiapoptotic protein, Bcl-2, in porcine kidney LLC-PK(1) cells. *J. Pharmacol. Exp. Ther.* **290,** 923–928.
35. Leccia, M. T., Richard, M. J., Favier, A., and Béani, J. C. (1999) Zinc protects against ultraviolet A1-induced DNA damage and apoptosis in cultured human fibroblasts. *Biol. Trace Elem. Res.* **69,** 177–190.
36. Manev, H., Kharlamov, E., Uz, T., Mason, R. P., and Cagnoli, C. M. (1997) Characterization of zinc-induced neuronal death in primary cultures of rat cerebellar granule cells. *Exp. Neurol.* **146,** 171–178.
37. Iguchi, K., Hamatake, M., Ishida, R., Usami, Y., Adachi, T., Yamamoto, H., Koshida, K., Uchibayashi, T., and Hirano, K. (1998) Induction of necrosis by zinc in prostate carcinoma cells and identification of proteins increased in association with this induction. *Eur. J. Biochem.* **253,** 766–770.
38. Telford, W. G., and Fraker, P. J. (1998) Zinc induced apoptosis in bone marrow and splenic B-lineage lymphocytes of the mouse. *Nutr. Res.* **18,** 319–326.
39. Wang, W., Post, J. I., Dow, K. E., Shin, S. H., Riopelle, R. J., and Ross, G. M. (1999) Zinc and copper inhibit nerve growth factor-mediated protection from oxidative stress-induced apoptosis. *Neurosci. Lett.* **259,** 115–118.
40. Zalewski, P. D., Forbes, I. J., and Betts, W. H. (1993) Correlation of apoptosis with change in intracellular labile Zn(II) using zinquin [(2-methyl-8-*p*-toluenesulphonamido-6-quinolyloxy)acetic acid], a new specific fluorescent probe for Zn(II). *Biochem. J.* **296,** 403–408.
41. Zalewski, P. D., Forbes, I. J., Seamark, R. F., Borlinghaus, R., Betts, W. H., Lincoln, S. F., and Ward, A. D. (1994) Flux of intracellular labile zinc during apoptosis (gene-directed cell death) revealed by a specific chemical probe, Zinquin. *Chem. Biol.* **1,** 153–161.
42. Duffy, J. Y., Miller, C. M., Rutschilling, G. L., Ridder, G. M., Clegg, M. S., Keen, C. L., and Daston, G. P. (1999) Decreased intracellular zinc levels alter mitochondrial membrane function and increase caspase-3 activity in HL-60 cells. *Toxicol. Sci.* **48,** 307.
43. Nicholson, D. W., and Thornberry, N. A. (1997) Caspases: killer proteases. *Trends Biochem. Sci.* **22,** 299–306.
44. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labele, M., Lazebnik, Y. A., *et al.* (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis [see comments]. *Nature* **376,** 37–43.
45. Tewari, M., Quan, L. T., K, O. R., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell.* **81,** 801–809.
46. Perry, D. K., Smyth, M. J., Stennicke, H. R., Salvesen, G. S., Duriez, P., Poirier, G. G., and Hannun, Y. A. (1997) Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis. *J. Biol. Chem.* **272,** 18530–18533.
47. Stennicke, H. R., and Salvesen, G. S. (1997) Biochemical characteristics of caspases-3, -6, -7, and -8. *J. Biol. Chem.* **272,** 25719–25723.
48. Humanson, G. L. (1979) Solution preparation. *In* Animal Tissue Techniques, pp. 541–563.
49. Sulik, K. K., Cook, C. S., and Webster, W. S. (1988) Teratogens

and craniofacial malformations: Relationships to cell death. *Development* **103**(Suppl.), 213–232.

50. Jankowski-Hennig, M. A., Clegg, M. S., Daston, G. P., Rogers, J. M., and Keen, C. L. (1999) Zinc deficiency-induced cell death in rat embryos is associated with increased caspase 3-like activity. *FASEB J.* **13,** A570.
51. Clegg, M. S., Keen, C. L., Lonnerdal, B., and Hurley, L. S. (1981) Influence of ashing techniques on the analysis of trace elements in animal tissue. *Biol. Trace Elements Res.* **3,** 107–115.
52. SAS Institute (1992–1998) Statview for Windows, 5.0.1 ed.
53. Taubeneck, M. W., Daston, G. P., Rogers, J. M., and Keen, C. L. (1994) Altered maternal zinc metabolism following exposure to diverse developmental toxicants. *Reprod. Toxicol.* **8,** 25–40.
54. Rogers, J. M., Lonnerdal, B., Hurley, L. S., and Keen, C. L. (1987) Iron and zinc concentrations and ^{59}Fe retention in developing fetuses of zinc-deficient rats. *J. Nutr.* **117,** 1875–1882.
55. Fraker, P. J., Haas, S. M., and Luecke, R. W. (1977) Effect of zinc deficiency on the immune response of the young adult A/J mouse. *J. Nutr.* **107,** 1889–1895.
56. Aiuchi, T., Mihara, S., Nakaya, M., Masuda, Y., Nakajo, S., and Nakaya, K. (1998) Zinc ions prevent processing of caspase-3 during apoptosis induced by geranylgeraniol in HL-60 cells. *J. Biochem. (Tokyo)* **124,** 300–303.
57. Clegg, M. S., Hong, H., Trapp, C., Duffy, J., Daston, G., and Keen, C. L. (1997) Induction of caspase 3 activity in 3T3 cells cultured in zinc deficient medium. *FASEB J.* **12,** A522.
58. DiSilvestro, R. A., and Blostein-Fujii, A. (1997) Moderate zinc deficiency in rats enhances lipoprotein oxidation *in vitro*. *Free Radical Biol. Med.* **22,** 739–742.
59. Olin, K. L., Shigenaga, M. K., Ames, B. N., Golub, M. S., Gershwin, M. E., Hendrickx, A. G., and Keen, C. L. (1993) Maternal dietary zinc influences DNA strand break and 8-hydroxy-2′-deoxyguanosine levels in infant rhesus monkey liver. *Proc. Soc. Exp. Biol. Med.* **203,** 461–466.
60. Olin, K. L., Golub, M. S., Gershwin, M. E., Hendrickx, A. G., Lonnerdal, B., and Keen, C. L. (1995) Extracellular superoxide dismutase activity is affected by dietary zinc intake in nonhuman primate and rodent models. *Am. J. Clin. Nutr.* **61,** 1263–1267.
61. Oteiza, P. I., Olin, K. L., Fraga, C. G., and Keen, C. L. (1995) Zinc deficiency causes oxidative damage to proteins, lipids and DNA in rat testes. *J. Nutr.* **125,** 823–829.
62. Buttke, T. M., and Sandstrom, P. A. (1994) Oxidative stress as a mediator of apoptosis [see comments]. *Immunol. Today* **15,** 7–10.
63. Oteiza, P. I., Clegg, M. S., Zago, M. P., and Keen, C. L. (2000) Zinc deficiency induces oxidative stress and AP-1 activation in 3T3 cells. *Free Radical Biol. Med.*, in press.
64. Cartwright, M. M., Tessmer, L. L., and Smith, S. M. (1998) Ethanol-induced neural crest apoptosis is coincident with their endogenous death, but is mechanistically distinct. *Alcohol Clin. Exp. Res.* **22,** 142–149.
65. Chen, S. Y., and Sulik, K. K. (1996) Free radicals and ethanol-induced cytotoxicity in neural crest cells. *Alcohol Clin. Exp. Res.* **20,** 1071–1076.
66. DePasquale-Jardieu, P., and Fraker, P. J. (1979) The role of corticosterone in the loss in immune function in the zinc-deficient A/J mouse. *J. Nutr.* **109,** 1847–1855.
67. DePasquale-Jardieu, P., and Fraker, P. J. (1980) Further characterization of the role of corticosterone in the loss of humoral immunity in zinc-deficient A/J mice as determined by adrenalectomy. *J. Immunol.* **124,** 2650–2655.
68. Fraker, P. J., Osati-Ashtiani, F., Wagner, M. A., and King, L. E. (1995) Possible roles for glucocorticoids and apoptosis in the suppression of lymphopoiesis during zinc deficiency: A review. *J. Am. Coll. Nutr.* **14,** 11–17.
69. Telford, W. G., and Fraker, P. J. (1997) Zinc reversibly inhibits steroid binding to murine glucocorticoid receptor. *Biochem. Biophys. Res. Commun.* **238,** 86–89.