Zinc-Silicon Interactions Influencing Sperm Chromatin Integrity and Testicular Cell Development in the Rat as Measured by Flow Cytometry[1]

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ABSTRACT: Flow-cytometric procedures were used to determine effects of dietary Zn and Si variations on rat testicular cell development, including integrity of caudal epididymal sperm chromatin structure defined as the susceptibility of DNA to denaturation *in situ*. Concentrations of 4 (deficient), 12 (adequate), and 500 (excessive) mg of Zn/kg of diet were used with Si concentrations of 0 (low), 540 (medium), and 2,700 (high) mg/kg of diet in a 3 × 3 factorial arrangement. Three-week-old Sprague-Dawley male rats were fed the experimental diets for 8 wk. Rats fed the Zn-deficient/Si-low diet demonstrated significant deviations in the ratio of testicular cell types present, including a reduction of S phase and total haploid cells. Furthermore, approximately 50% of epididymal sperm had a significant decrease in resistance to DNA denaturation *in situ*. In the Zn-deficient/Si-medium treatment, the effects of Si on animal and testicular growth, distribution of testicular cell types, and sperm chromatin structure integrity were quite similar to the effects of the Zn-adequate diets. A toxic effect of Zn on sperm chromatin structure integrity observed in the Zn-excess/Si-medium treatment seemed to be counteracted by Si in the Zn-excess/Si-high treatment. Silicon at medium and high levels seems to affect Zn metabolism through potentiation and antagonistic reactions, respectively. Zinc deficiency likely disrupts the normal sperm chromatin quaternary structure in which Zn plays a role by providing stability and resistance to DNA denaturation *in situ*.

Key Words: Zinc, Silicon, Flow Cytometry, Spermatogenesis

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Introduction

Zinc is almost ubiquitously distributed in biological tissues and serves many vital roles, including an important role in mammalian spermatogenesis, as reviewed by Hidiroglou and Knipfel (1984). Rats raised on a Zn-deficient diet are noted for having reduced body and testicular weight, which, if severe, produces irreversible damage to seminiferous tubules and sterility (Millar et al., 1958).

Zinc is known to play a role in the stabilization of mammalian sperm chromatin structure, apparently by complexation with –SH groups of protamines (Huret, 1986; Kvist and Eliasson, 1978; Gatewood, 1988). However, the temporal role of Zn stabilization during epididymal maturation, the accumulation of zinc from Zn-rich prostatic fluid (Mann, 1964; Kvist et al., 1985), and the loss of zinc before fertilization (Kvist, 1980) is not well understood. Human ejaculated sperm contain approximately one Zn atom per protamine molecule (Gatewood, 1988). Evenson et al. (1980) have shown that a Zn-deficient diet caused destabilization of mouse caudal epididymal sperm chromatin structure.

Silicon has been shown to be an essential element for growth of chicks (Carlisle, 1972) and rats (Schwarz and Milne, 1972). Working with Zn-deficient rats, Emerick and Kayongo-Male (1990) reported an apparent antagonism between Si and Zn, whereby elevated levels of one of these elements reduced blood concentrations of the other. A subsequent study (Stewart et al., 1993) revealed a Si-Zn interaction in which a medium level of Si (540 mg/kg of diet) seemed to potentiate the metabolic activity of Zn, whereas a high level (2,700 mg/kg of diet) seemed to lower Zn concentrations. Although dietary Si at

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concentrations of 250 mg/kg for chicks and 500 mg/kg for rats was used to demonstrate Si essentiality, 2,700 mg/kg has been used extensively by Emerick and coworkers (Emerick, 1984, 1986; Schreier and Emerick, 1986; Emerick and Lu, 1987) to promote silica urolithiasis in rats.

This study was based on the hypothesis that rats fed a Zn-deficient diet would demonstrate an altered pattern of spermatogenesis and an altered sperm chromatin structure, as defined by an increased susceptibility to DNA denaturation *in situ*. In addition, it was hypothesized that medium levels of Si would help overcome the adverse effects of a Zn-deficient diet.

Experimental Procedures

Testes and cauda epididymal sperm were obtained from the rats used for Exp. 1 in the studies described by Stewart et al. (1993). The basal diet was a dextrose-egg albumin type, in which the spray-dried egg albumin was first denatured by autoclaving and then dried, each at 120°C for 1 h, to reduce avidin interference with biotin absorption. Tetraethylorthosilicate (J. T. Baker Chemical, Phillipsburg, NJ), added at the expense of dextrose, provided Si above the 5 mg/kg inherent in the basal diet, and added dietary Zn, above the .25 mg/kg inherent in the basal diet, was provided by $ZnCl_2$ (J. T. Baker Chemical), both determined by flame atomic absorption spectrophotometric measurements (Emerick and Kayongo-Male, 1990). Added Zn concentrations at 4 (Zn-deficient), 12 (Zn-adequate), and 500 (Zn-excess) mg/kg of diet and added Si concentrations of 0 (Si-low), 540 (Si-medium), and 2,700 (Si-high) mg/kg of diet were used in 3 × 3 factorial arrangement.

Male Sprague-Dawley rats 3 wk of age were obtained from SASCO (Omaha, NE) and individually housed in hanging stainless steel cages with wire mesh floors. The rats were given ad libitum access to diets in glass jars, and distilled water was provided in polyethylene bottles fitted with stainless steel drinking tips. The experiment involved 10 rats per treatment for Si-low or Si-medium treatments and 20 per treatment for the Si-high treatments.

After 8 wk on the experimental regimen, the rats were killed by decapitation while they were anesthetized with Halothane (Abbott Laboratories, Chicago, IL). Testicular germ cells and epididymal sperm were obtained, as described below, from 10 animals per treatment. The first 10 animals killed from the Si-high group were used for experiments described here.

Testicular Germ Cells

Testes were surgically removed and a portion minced with curved scissors into a cellular suspension in 60-mm Petri dishes containing 2 mL of Hanks balanced salt solution (Gibco, Grand Island, NY) at 4°C and then transferred to 12-mm × 75-mm polystyrene tubes. After settling of tissue fragments, the supernatants were filtered through 53-μm nylon mesh (Tetko, New York) into 12-mm × 75-mm tubes and kept on crushed ice until measured by flow cytometry (**FCM**). Measurements by FCM were completed within approximately 20 min of euthanasia.

Epididymal Sperm

Cauda epididymi were surgically removed and placed in a 60-mm Petri dish containing 2.0 mL of TNE buffer (.15 *M* NaCl, .01 *M* Tris-HCl, .001 *M* EDTA, pH 7.4) at 4°C. Each epididymis was sliced several times with a single-edged razor blade and minced with curved scissors. The sperm suspensions were gently aspirated and expelled several times through Pasteur pipettes and filtered through 153-μm nylon mesh into 12-mm × 75-mm tubes. Each sperm sample was placed in a 2.0-mL snap-cap tube, frozen at −20°C for 2 h, and stored at −100°C.

Cell Staining with Acridine Orange

Fresh testicular or frozen/thawed (37°C) epididymal sperm aliquots (.20 mL) diluted to a concentration of 1 to 2 × 10^6/mL were admixed with .40 mL of .1% Triton X-100, .15 *M* NaCl, and .08 *N* HCl, pH 1.4. Triton X-100 makes cell membranes permeable, providing accessibility to DNA for acridine orange (**AO**) staining. After 30 s, the cells were stained by adding 1.2 mL of solution containing 6.0 μg of chromatographically purified AO (Polysciences, Warrington, PA) per milliliter of AO buffer (370 mL of .1 *M* citric acid and 630 mL of .2 *M* Na_2HPO_4, 1 m*M* EDTA, .15 *M* NaCl, pH 6.0; Darzynkiewicz et al., 1976; Evenson et al., 1985). Measurements by FCM were completed within approximately 3 min after initiation of this procedure.

When excited by blue laser light in the flow cytometer, AO intercalated into native, double-stranded DNA fluoresces green; AO associated with single-stranded nucleic acid (DNA or RNA) emits red fluorescence, as reviewed by Darzynkiewicz and Kapuscinski (1990). The metachromatic fluorescence observed in AO-stained testicular cells reflects the relative content of DNA (green fluorescence) and RNA (red fluorescence; Darzynkiewicz et al., 1976; Evenson and Melamed, 1983). Because normal, mature sperm cells contain virtually no RNA (Monesi, 1965), red fluorescence is minimal (Evenson and Melamed, 1983). Normal isolated sperm nuclei and(or) whole cells treated with RNAse demonstrate the same fluorescence pattern as whole cells, indicating that the fluorescence is due to DNA staining (Evenson et al., 1985; Ballachey et al., 1987).

Table 1. Rat body weights (BW), testes weights, and distribution of testicular cells[a]

Dietary treatment, mg/kg				Distribution of testicular cells										
				Cell type, %										
					Diploid			Tetraploid			Haploid cell type, %			
Zn	Si	BW, g[b]	Testes wt, g	Haploid	All	Box 4	Box 3	All	Box 2	Box 1	Round	Elongating	Elongated	Cycling,%
4	0	242[f]	2.67[f]	66.8[f]	25.1[f]	21.3[f]	3.9	8.1[h]	4.3	3.7[f]	50.7[h]	19.9	29.4[f]	6.8[f]
4	540	297[g]	3.42[g]	80.8[g]	12.2[g]	8.7[g]	3.5	7.0[i]	4.2	2.9[g]	42.3[i]	22.3	35.4[g]	11.5[g]
4	2,700	231[f]	2.70[f]	78.5[g]	13.1[g]	9.3[g]	3.8	8.5[h]	4.9	3.6[f]	45.8[i]	20.1	34.1[g]	8.4[f]
12	0	292	3.34	81.8	11.6	8.2	3.4	6.6	3.9	2.7[h]	42.2	21.9	36.0	11.1
12	540	291	3.33	79.7	12.8	9.2	3.6	7.5	4.3	3.2[i]	43.2	20.9	35.9	10.4
12	2,700	292	3.19	80.9	11.9	8.8	3.1	7.2	4.4	2.8[h]	40.7	22.7	36.6	10.1
500	0	319[f]	3.48[hi]	80.8	12.1	9.1	3.0	7.1	4.2	2.9	42.8	22.1	35.1	11.4[f]
500	540	301[fg]	3.52[h]	81.1	11.6	8.3	3.3	7.3	4.1	3.1	44.8	22.2	33.0	10.0[fg]
500	2,700	285[g]	3.28[i]	80.9	11.6	8.2	3.4	7.5	4.4	3.1	45.7	20.3	34.0	8.5[g]
Overall Zn effect[c]														
4	—	—	—	—	—	—	3.7[h]	7.8	4.5	—	20.8	—	—	—
12	—	—	—	—	—	—	3.4[i]	7.1	4.2	—	21.8	—	—	—
500	—	—	—	—	—	—	3.2[i]	7.3	4.2	—	21.6	—	—	—
Overall Si effect[c]														
—	0	—	—	—	—	—	3.4	7.3	4.1	—	21.3	—	—	—
—	540	—	—	—	—	—	3.4	7.3	4.2	—	21.8	—	—	—
—	2,700	—	—	—	—	—	3.5	7.7	4.6	—	21.1	—	—	—
SEM[d]		8.0	.10	1.6	1.6	1.6	.2	.3	.3	.2	1.5	1.2	1.0	.8
GLM[e]														
Zn		< .01	< .01	< .01	.01	< .01	.02	.05	NS	.01	< .01	NS	< .01	.06
Si		< .01	< .01	.01	<.01	< .01	NS	NS	NS	NS	NS	NS	NS	.03
Zn × Si		< .01	< .01	< .01	<.01	< .01	NS	.07	NS	.01	.02	NS	< .01	< .01

[a]n = 10 rats/treatment except for BW and testes weight, where n = 20 rats for treatments with 2,700 mg of Si/kg of diet.
[b]Average initial weight = 53.4 g (SEM = 1.4).
[c]Presentation of overall Zn or Si effects is precluded when a significant Zn × Si interaction exists.
[d]Standard error of the mean calculated from the error mean square.
[e]A 3 × 3 analysis using Type III sums of squares; numerical values are levels of probability for main effects and interaction; NS = not significant at $P \geq .10$.
[f,g]Least squares means within a column and the same Zn treatment or overall effect lacking a common superscript letter differ ($P < .01$).
[h,i]Least squares means within a column and the same Zn treatment or overall effect lacking a common superscript letter differ ($P < .10$).

Sperm Chromatin Structure Assay

When used on sperm, the procedure involving AO staining and subsequent FCM measurements has been termed the sperm chromatin structure assay (**SCSA**; Evenson, 1989, 1990). Even though the low pH treatment does not cause denaturation of histone-complexed DNA in sperm, it apparently does cause partial DNA denaturation in sperm having altered chromatin structure (Evenson et al., 1985). Abnormal chromatin structure, defined here as an increased susceptibility to acid-induced denaturation, is determined by flow cytometric measurements of the shift from green (native DNA) to red (denatured, single-stranded DNA) fluorescence. This shift is expressed by alpha t (α_t; Darzynkiewicz et al., 1975) and is the ratio of red to total (red + green) fluorescence.

In the SCSA, α_t is calculated for each sperm, and results are expressed for each sample as the mean ($\mathbf{x}\alpha_t$), standard deviation ($\mathbf{SD}\alpha_t$), and percentage of cells outside the main population of α_t ($\mathbf{COMP}\alpha_t$). Measurement of normal sperm produces a very narrow α_t distribution, but a sperm population with denatured DNA has a broader distribution, hence larger $SD\alpha_t$ and $COMP\alpha_t$.

Flow Cytometric Measurements

A Cytofluorograf II flow cytometer interfaced to a 2150 computer (Ortho Diagnostic Systems, Westwood, MA) equipped with ultrasense optics and a Lexel 100 mW argon ion laser operated at 35 mW and producing a 488 nm (blue) excitation wavelength was used to measure the cells. Dual parameter FCM measurements were made to determine amounts of 1) DNA vs RNA in testicular cells and 2) double-stranded (native) vs single-stranded (denatured) DNA in sperm cells. Total fluorescence from each cell in the sample population was separated into green (515 to 530 nm) and red (> 630 nm) components. The computer calculated α_t values for each of 5,000 cells in each sample population.

Data Analysis

Data were analyzed by the GLM procedure (SAS, 1988) using a model consisting of levels of Zn and Si as main effects and the two-way interaction. The method of least significant differences protected by a significant *F*-value (FLSD, Steel and Torrie, 1980) was used to identify significant differences among the three levels of a factor. In the presence of a significant

Zn × Si interaction, Si levels were nested within Zn levels for analysis. Differences were considered statistically significant at $P < .05$.

Results and Discussion

Data pertaining to BW, testes weights, and distribution of testicular cell types are shown in Table 1. Although previously presented by Stewart et al. (1993), BW are included here to simplify data interpretation. Average BW was low in Zn-deficient/Si-low rats and was 23% higher ($P < .01$) in the Zn-deficient/Si-medium treatment. This Si-mediated weight-gain response was postulated by Stewart et al. (1993) to be due to a potentiation of the metabolic activity of Zn. The Zn-deficient/Si-high treatment also reduced BW by 29% ($P < .01$) compared with the Zn-deficient/Si-medium treatment.

Testes weights (grams/rat, Table 1) showed approximately the same relative differences in BW, but when calculated as a percentage of BW, testes weights did not differ among treatments. Zinc concentration of the testes from the Zn-deficient rats, as detailed by Stewart et al. (1993), was only 89% of the value of Zn-adequate rats ($P < .01$). Similarly, mean testis Zn concentration of Si-low rats was only 94% ($P < .01$) of the value of Si-medium rats, and it approximated that of Zn-deficient rats. Conversely, the mean testis Zn concentration for Si-high treatments also approximated that of Zn-deficient rats. Retardation of testis development has been reported to result from the feeding of Zn-poor diets to several species, including the rat (Gunn and Gould, 1970).

Dual parameter (DNA vs RNA) flow cytometry measurements of AO-stained rat testicular cells provided the raw data shown in Figure 1. Each dot corresponds to a single cell and is located on the scattergram (cytogram) relative to its DNA and RNA content, except that within Boxes 5 to 7 the DNA content is the same for these haploid cells, but they differ in their stainability due to condensation of nuclear chromatin (Gledhill et al., 1966; Evenson et al., 1986b). Box 2 contains mostly pachytene spermatocytes that have not yet begun the large RNA increase characteristic of late pachytene and diplotene spermatocytes in Box 1 (Courot et al., 1970; Evenson et al., 1986a). Cells in Boxes 3 and 4 are mostly diploid germ cells and a very small percentage of somatic interstitial cells (Evenson et al., 1986a). These data show at a single time point a "snapshot" of the relative percentage of each cell type present in the testis of rats fed different diets. Cell progression through spermatogenesis is known to proceed from diploid (Boxes 3 and 4) to tetraploid cells (Boxes 1 and 2), through meiosis to haploid round spermatids (Box 5). This is followed by a loss of RNA (elongating spermatids, Box 6) and condensed nuclei with reduced

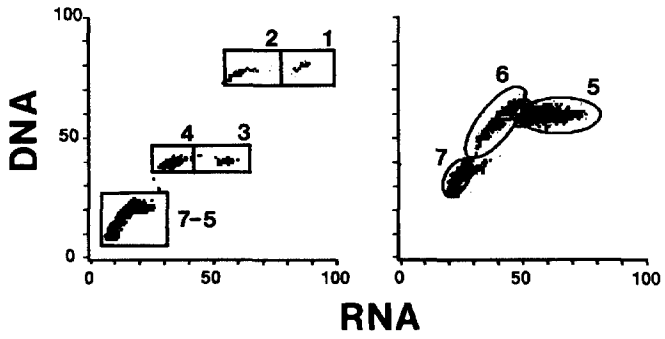

Figure 1. Dual-parameter cytogram of green fluorescence (DNA stainability) vs red fluorescence (RNA stainability) of rat testicular cells. The units on the x and y axes correspond to relative, linear fluorescence intensity. Boxes 1–2, 3–4, and 5–7 correspond to tetraploid, diploid, and haploid cell types, respectively. The right panel is an enhancement of Boxes 5 to 7 in the left panel in order to resolve the three populations consisting of round (Box 5), elongating (Box 6) and elongated (Box 7) spermatids.

DNA stainability (Box 7). Therefore, an observed alteration of cell type distributions can be attributed to an agent (e.g., high- and low-Zn and -Si diets) that alters the normal kinetics of cell proliferation and differentiation. For example, an increase in one cell type can be due to a metabolic block that prevents that cell type from progressing to the next maturation stage. A loss of a cell type may be due to cell kill, or, more likely in this study, to a metabolic block imposed on an earlier cell stage that prevents the appearance of the next cell stage.

The relative percentage of total diploid cells was higher ($P < .01$) in the Zn-deficient/Si-low rats than in the Zn-deficient rats fed the two higher levels of Si (Table 1). The box notations in Table 1 refer to the same boxes as in Figure 1. This abnormal increase in diploid cells is due entirely to a higher percentage of cells with the lower RNA content. Table 1 also shows an increase ($P < .01$) in the percentage of the late pachytene and diplotene spermatocytes (Box 1). These data, together with the observation (Table 1) that testicular cells from Zn-deficient/Si-low rats had a lower percentage of DNA synthesizing cells (S-phase cycling; 6.8 vs 11.5%; $P < .01$) than those from Zn-deficient/Si-medium rats, strengthen the argument that Zn deficiency inhibited DNA synthesis and(or) entry into S-phase, perhaps due to inhibition of Zn-dependent cell proliferative activities (e.g., DNA and RNA polymerase activity; Prasad, 1977). Such an effect could cause an accumulation of diploid germ cells in the G1 phase of the cycle; these cells likely correspond to a large increase (21.3 vs 8.7%; $P < .01$) in the cells in Box 4 that contain low RNA. Zinc-deficient/Si-high rats also had a lower ($P < .01$)

percentage of cycling cells than did Zn-deficient/Si-medium rats.

In addition to the apparent inhibitory effect on DNA synthesis, Zn deficiency may have also inhibited the meiotic process, because the percentage of cells in Box 1 (precursor cell to daughter meiotic cells) of the Zn-deficient/Si-low group is elevated; it is during the pachytene stage of the meiotic prophase that cells seem to be particularly susceptible to many forms of damage (Setchell, 1978). The end result of inhibition of DNA synthesis and meiosis is the reduced percentage of total haploid cells (66.8 vs 80.8%; $P < .01$) in Zn-deficient/Si-low vs Zn-deficient/Si-medium treatments. Rats fed either a Zn-adequate or Zn-excess diet with various levels of Si did not demonstrate an abnormal testicular cell type ratio. An exception to the latter is the lower percentage (8.5 vs 11.4%; $P < .01$) of S-phase cycling cells in Zn-excess/Si-high rats than in Zn-excess/Si-low rats.

The Zn-deficient/Si-low diet caused not only a disturbance of spermatogenesis (production of meiotic daughter cells), but also differentiation of meiotic daughter cells into late-stage spermatids. Rats fed a Zn-deficient/Si-low diet, compared with Zn-deficient groups with higher levels of Si, had a slightly higher relative percentage of round spermatids ($P < .10$; Table 1). This suggests that Zn deficiency, most prevalent in the absence of added dietary Si, inhibited differentiation. A corresponding decrease of approximately the same magnitude in the relative percentage of elongated spermatids may be a result of a slowed progression of cells through spermiogenesis ($P < .01$; Table 1). With the exception of the Zn-deficient/Si-low diet, all other combinations of diet had no significant effect on kinetics of spermiogenesis. Stewart et al. (1993) found rats fed the Si-high diets to have lower plasma alkaline phosphatase activity than those fed Si-medium diets. Also, the Zn-deficient/Si-high animals had lower heart Zn concentrations. This indicates that Zn deficiency was the principal factor causing the greatest testicular cell aberrations in the current study.

During mammalian spermiogenesis, histones associated with round spermatid DNA are replaced by transition proteins, and finally by protamines (Grimes et al., 1977). In mature rat sperm, the nucleoprotein consists of a single protamine species, designated S1 (Bellve et al., 1975). Protamines are small, arginine-rich, structural proteins that stabilize sperm DNA in an insoluble nucleoprotamine complex. Zinc is known to play a role in stabilization of sperm chromatin structure (Kvist and Eliasson, 1978; Gatewood, 1988). Balhorn (1982) has presented a model of protamine binding in the minor groove of the DNA with protrusions of the protamines lying in the major grooves of adjacent DNA molecules. Calvin et al.

Table 2. Rat sperm chromatin structure integrity as measured by flow cytometry[a]

Dietary treatment, mg/kg		Sperm chromatin structure assay (± SD)		
Zn	Si	$X\alpha_t$[b]	$SD\alpha_t$[c]	$COMP\alpha_t$[d]
4	0	269^{g} ± 79	70^{g} ± 69	52^{g} ± 32
4	540	206^{h} ± 10	19^{h} ± 3	19^{h} ± 16
4	2,700	250^{g} ± 51	52^{g} ± 47	58^{g} ± 23
12	0	198 ± 12	20 ± 3	8 ± 13
12	540	192 ± 6	16 ± 2	3 ± 2
12	2,700	200 ± 14	20 ± 5	14 ± 23
500	0	203 ± 9	18 ± 5	14^{i} ± 18
500	540	213 ± 29	28 ± 16	28^{j} ± 29
500	2,700	201 ± 15	20 ± 7	18^{ij} ± 25
GLM[ef]				
Zn		< .01	< .01	< .01
Si		.10	NS	.08
Zn × Si		.02	.02	< .01

[a]n = 10 rats/treatment.

[b]α_t = alpha t, the ratio of red to total (red + green) fluorescence, a measure of chromatin structure abnormality within a population.

[c]$SD\alpha_t$ = standard deviation of α_t.

[d]$COMP\alpha_t$ = cells outside of main population of α_t.

[b,c,d]Although α_t values range from 0 to 1 by definition, they are × 10^3 here for ease of whole-number manipulation.

[e]A 3 × 3 analysis using Type III sums of squares; numerical values are levels of probability for main effects and interaction; NS = not significant at $P \geq .10$.

[f]Presentation of overall Zn or Si effects is precluded when a significant Zn × Si interaction exists.

[g,h]Least squares means within a column and the same Zn treatment or overall effect lacking a common superscript letter differ ($P < .01$).

[i,j]Least squares means within a column and the same Zn treatment or overall effect lacking a common superscript letter differ ($P < .05$).

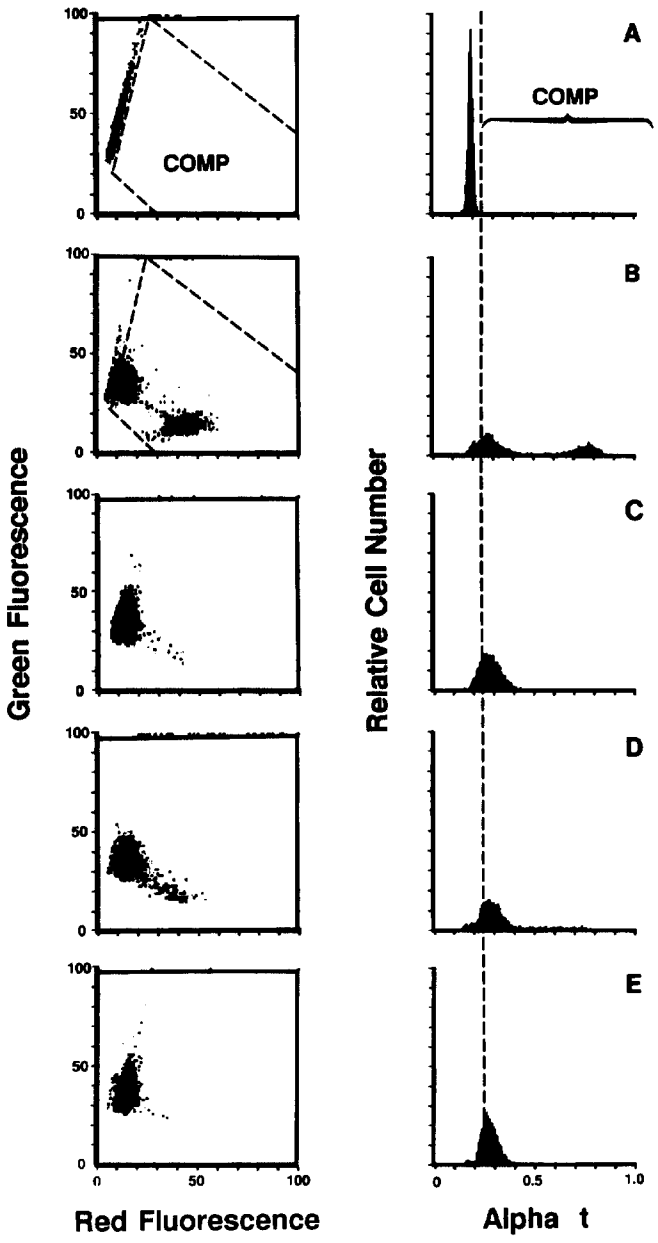

Figure 2. The left panel shows dual parameter cytograms of green fluorescence (native DNA) vs red fluorescence (denatured DNA) of rat sperm treated by the SCSA. The units on the x and y axes correspond to relative, linear fluorescence intensity. The panel on the right shows α_t frequency histograms derived from the raw data in the left panels. Alpha t has a range from 0 to 1.0. Panel A is from normal rat sperm showing virtually no cells outside the main population. Panels B and C are derived from rats fed Zn-deficient/Si-low diet and panels D and E are derived from rats fed Zn-deficient/Si-high diets.

(1973) suggested that high levels of cysteine in mammalian protamines allowed for cross-linking of mammalian sperm chromatin. These authors demonstrated that mammalian sperm were resistant to solubilization in sodium dodecyl sulfate unless dithiothreitol was added to the detergent solution. In addition, they noted that inclusion of .1 m*M* EDTA resulted in a 20% increase in free sulfhydryl groups, and the inclusion of Zn inhibited the labeling of the –SH groups.

Flow cytometric analysis of cauda epididymal sperm by the SCSA provides solid evidence that Zn deficiency causes significant perturbations of normal sperm chromatin development. Figure 2 shows representative data for susceptibility of DNA to denaturation *in situ*. Figure 2A depicts a normal pattern with an elliptical scattergram distribution for green and red fluorescence resulting in α_t frequency distribution that has a very small CV and SD. Normal populations of rat sperm have a low percentage, approximately 5% or less, of cells outside the main population of α_t (COMPα_t). However, the data in Figure 2B and Table 2 demonstrate a very pronounced effect of Zn deficiency on integrity of sperm chromatin structure, similar to that observed in our previous study with mice (Evenson et al., 1980). Cytogram patterns varied from normal (Figure 2A) to populations that shifted heterogeneously (Figure 2B,D) or more homogeneously (Figure 2C,E). Not only were all the α_t variables significantly elevated for the Zn-deficient/Si-low and Zn-deficient/Si-high vs the Zn-deficient/Si-medium group, but the standard deviations are very high, indicating heterogeneity of response among the animals. For example, Zn-deficient/Si-low rats had a COMPα_t of 52 ± 32, corresponding to an approximate 50:50 ratio of normal to abnormal sperm. The Zn-deficient/Si-medium rats showed a more normal development of sperm chromatin structure. Although the mean and standard deviations of α_t for this group were relatively normal, the COMPα_t at 19 ± 16 was considered to be elevated. The Zn-deficient/Si-high diet had a deleterious effect on sperm chromatin structure almost equivalent to that of the Zn-deficient/Si-low diet.

Zinc-adequate treatments, regardless of Si level, showed no major effects on chromatin structure, with the exception of a relatively high COMPα_t that had high within-treatment variation for the Zn-adequate/Si-high rats.

Excess Zn had a negative effect, but much less than that of Zn deficiency, on sperm chromatin structure, as noted by the relatively high COMPα_t values, which ranged from 14 ± 18 to 28 ± 29 for the Zn-excess treatments (Zn effect, $P < .01$; Zn × Si interaction, $P < .01$). The Zn-excess/Si-medium treatment had a higher ($P < .05$) COMPα_t than the Zn-excess/Si-low treatment. This supports the conclusion that the 540 mg of Si/kg of diet (Si-medium) potentiated the metabolic activity of Zn even when Zn was present at a potentially toxic level. It should be noted that COMPα_t measurements are more sensitive to detecting abnormalities when the majority of sperm react

homogeneously to treatment (e.g., Figure 2C,E) . A relatively high degree of homogeneity has been seen in studies on bull sperm in our laboratory (Ballachey et al., 1987), whereas most of our rodent toxicology (Evenson et al., 1985, 1986a, 1989b,c; Evenson, 1990) and human (Evenson et al., 1991) studies have demonstrated greater heterogeneity among sperm in a population. In instances of high heterogeneity, $SD\alpha_t$ has been the most sensitive parameter to detect chromatin structure abnormalities. The observation that Zn-high diets cause abnormalities of chromatin structure needs more investigation, but it is possible that this potentially toxic concentration of Zn destabilizes S-S bonds and Zn complexation within and between protamine molecules, leading to a destabilization of sperm chromatin quaternary structure and greater susceptibility to DNA denaturation *in situ*.

In contrast to some other studies on ejaculated sperm that have been in contact with Zn-rich prostatic fluid (Kvist et al., 1985), these studies have been conducted on caudal epididymal sperm. Thus, Si-mediated benefits are effected before ejaculation. Rat caudal sperm nuclei have 7% more free-SH groups than vas sperm nuclei (Evenson et al., 1989a). Therefore, the Zn complexation pattern in caudal sperm may be different from vas or ejaculated sperm.

These data, additional to data detailed by Stewart et al. (1993), provide support for the concept that Si potentiates the metabolic activity of Zn. In rats fed the Zn-deficient diet, the Si-medium treatment provided benefits regarding growth, distribution of testicular cells, and sperm chromatin structure integrity equivalent to those derived from increasing dietary Zn to the Zn-adequate level. Also, a possible Si-Zn antagonism regarding sperm chromatin integrity was seen, in which the Si-excess treatment seemed to counteract the effect of the Zn-adequate treatment.

The susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ* has been shown to be inversely related to bull fertility ranking (Evenson et al., 1980; Ballachey et al., 1987, 1988) and shows a dose response to toxic chemicals (Evenson et al., 1985; Evenson, 1989). The data presented here for both Zn-deficient and Si-high diets are similar to those seen in response to toxic chemicals (Evenson et al., 1989a,b). Furthermore, abnormalities observed in chromatin structure for Zn-deficient and Si-high diets are consistent with male infertility.

Implications

This study demonstrates that a Zn-deficient diet has negative effects on testicular function and sperm chromatin structure of rats. These effects are countered by addition of dietary Si, which probably interacts by potentiation and antagonistic mechanisms, respectively. A high-Si diet also disrupts spermatogenesis, but the effect seems to be less than that attributed to a Zn-deficient diet. Appropriate dietary Zn levels are important for efficient breeding potential.

Literature Cited

Balhorn, R. 1982. A model for the structure of chromatin in mammalian sperm. J. Cell Biol. 93:2998.

Ballachey, B. E., D. P. Evenson, and R. G. Saacke. 1988. The sperm chromatin structure assay. Relationship with alternate tests of semen quality, and heterospermic performance of bulls. J. Androl. 9:109.

Ballachey, B. E., W. D. Hohenboken, and D. P. Evenson. 1987. Heterogeneity of sperm nuclear chromatin structure and its relationship to bull fertility. Biol. Reprod. 36:915.

Bellve, A. R., E. Anderson, and L. Henley-Bowdoin. 1975. Synthesis and amino acid composition of basic proteins in mammalian sperm nuclei. Dev. Biol. 349.

Calvin, H. I., C. C. Yu, and J. M. Bedford. 1973. Effects of epididymal maturation, zinc (II) and copper (II) on the reactive sulfhydryl content of structural elements in rat spermatozoa. Exp. Cell Res. 81:333.

Carlisle, E. M. 1972. Silicon an essential element for the chick. Science 178:619.

Courot, M., M. T. Hochereau-De Reviers, and R. Ortavant. 1970. Spermatogenesis. In: A. D. Johnson, W. R. Gomes, and N. L. Vandemark (Ed.) The Testis. Vol. 1. pp 339–411. Academic Press, New York.

Darzynkiewicz, Z., and J. Kapuscinski. 1990. Acridine orange: A versatile probe of nucleic acids and other cell constituents. In: M. R. Melamed, T. Lindmo, and M. L. Mendelsohn (Ed.) Flow Cytometry and Sorting (2nd Ed.). p 291. Wiley-Liss, New York.

Darzynkiewicz, Z., F. Traganos, T. Sharpless, and M. Melamed. 1975. Thermal denaturation of DNA *in situ* as studied by acridine orange staining and automated cytofluorometry. Exp. Cell Res. 90:411.

Darzynkiewicz, Z., F. Traganos, R. Sharpless, and M. Melamed. 1976. Lymphocyte stimulation: A rapid multiparameter analysis. Proc. Natl. Acad. Sci. USA 73:2881.

Emerick, R. J. 1984. Chloride and phosphate as impediments to silica urinary calculi in rats fed tetraethylorthosilicate. J. Nutr. 114:733.

Emerick, R. J. 1986. Animal age and duration of exposure to a urolithic diet as factors influencing silica urolithiasis incidence in an animal model. Nutr. Rep. Int. 34:907.

Emerick, R. J., and H. Kayongo-Male. 1990. Interactive effects of dietary silicon, copper and zinc in the rat. J. Nutr. Biochem. 1: 35.

Emerick, R. J., and D. Lu. 1987. A possible synergism of dietary phosphate and urine acidifying salts in preventing silica urolithiasis in a rat model. J. Nutr. 117:1603.

Evenson, D. P. 1989. Flow cytometry evaluation of male germ cells. In: A. Yen (Ed.) Flow Cytometry: Advanced Research and Clinical Applications. Vol. I. p 217. CRC Press, Boca Raton, FL.

Evenson, D. P. 1990. Flow cytometry assays of male fertility. In: Z. Darzynkiewicz and H. Crissman (Ed.) Methods in Cell Biology, Vol. 33: Flow Cytometry. p 401. Academic Press, New York.

Evenson, D. P., R. K. Baer, and L. K. Jost. 1989a. Flow cytometric analysis of rodent epididymal spermatozoal chromatin condensation and loss of free sulfhydryl groups. Mol. Reprod. Dev. 1: 283.

Evenson, D. P., R. K. Baer, and L. K. Jost. 1989b. Long term effects of triethylenemelamine exposure on mouse testis cells and sperm chromatin structure assayed by flow cytometry. J. Environ. Mol. Mutagenesis 14:79.

Evenson, D. P., R. K. Baer, L. K. Jost, and R. W. Gesch. 1986a. Toxicity of thiotepa on mouse spermatogenesis as determined

by dual parameter flow cytometry. Toxicol. Appl. Pharmacol. 82:151.

Evenson, D. P., Z. Darzynkiewicz, L. Jost, F. Janca, and B. Ballachey. 1986b. Changes in accessibility of DNA to various fluorochromes during spermatogenesis. Cytometry 7:45.

Evenson, D. P., Z. Darzynkiewicz, and M. R. Melamed. 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. Science (Washington DC) 240:1131.

Evenson, D. P., P. H. Higgins, D. Grueneberg, and B. E. Ballachey. 1985. Flow cytometric analysis of mouse spermatogenic function following exposure to ethylnitrosourea. Cytometry 6:238.

Evenson, D. P., F. C. Janca, L. K. Jost, R. K. Baer, and D. S. Karabinus. 1989c. Flow cytometric analysis of effects of 1,3-Dinitrobenzene on rat spermatogenesis. J. Toxicol. Environ. Health 28:81.

Evenson, D. P., L. K. Jost, R. K. Baer, T. W. Turner, and S. M. Schrader. 1991. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. Reprod. Toxicol. 5:115.

Evenson, D. P., and M. R. Melamed. 1983. Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. J. Histochem. Cytochem. 31:248.

Gatewood, J. M. 1988. A study of human sperm chromatin. Ph.D. Dissertation. Univ. of California, Davis.

Gledhill, B. L., M. P. Gledhill, R. Rigler, Jr., and N. R. Ringertz. 1966. Changes in deoxyribonucleoprotein during spermiogenesis in the bull. Exp. Cell Res. 41:652.

Grimes, S. R., M. L. Meistrich, R. D. Platz, and L. S. Hnilica. 1977. Nuclear protein transitions in rat testis spermatids. Exp. Cell Res. 110:31.

Gunn, S. A., and T. C. Gould. 1970. Cadmium and other mineral elements. In: A. D. Johnson, M. R. Gomez, and N. L. Vandemark (Ed.) The Testis. Vol. 3. Influencing Factors. p 377. Academic Press, New York.

Hidiroglou, M., and J. E. Knipfel. 1984. Zinc in mammalian sperm: A review. J. Dairy Sci. 67:1147.

Huret, J. L. 1986. Nuclear chromatin decondensation of human sperm: A review. Arch. Androl. 16:97.

Kvist, U. 1980. Importance of spermatozoal zinc as temporary inhibitor of sperm nuclear chromatin decondensation ability in man. Acta Phys. Scand. 109:79.

Kvist, U., L. Bjorndahl, S. Kjellberg, C. Lindholmer, and G. M. Roomans. 1985. Sperm nuclear zinc: Its prostatic origin and its significance for human fertility. Acta Phys. Scand. (Suppl. 542) 124:389.

Kvist, U., and R. Eliasson. 1978. Zinc dependent chromatin stability in human ejaculated spermatozoa. First Intl. Congr. of Androl., Barcelona. Int. J. Androl. 1(Suppl. I):178.

Mann, T. 1964. The Biochemistry of Semen and of the Male Reproductive Tract. Wiley, New York.

Millar, M. J., M. I. Fischer, P. V. Elcoate, and C. A. Mawson. 1958. The effects of dietary zinc deficiency on the reproductive system of male rats. Can. J. Biochem. Physiol. 36:557.

Monesi, V. 1965. Synthetic activities during spermatogenesis in the mouse: RNA and protein. Exp. Cell Res. 39:197.

Prasad, A. S. 1977. Zinc in human nutrition. Crit. Rev. Clin. Lab. Sci. 8:1.

SAS. 1988. SAS Procedures Guide (Release 6.03 Ed.). SAS Inst. Inc., Cary, NC.

Schreier, C. J., and R. J. Emerick. 1986. Diet calcium carbonate, phosphorus and acidifying and alkalizing salts as factors influencing silica urolithiasis in rats fed tetraethylorthosilicate. J. Nutr. 116:823.

Schwarz, K., and D. B. Milne. 1972. Growth-promoting effects of silicon in rats. Nature (Lond.) 239:333.

Setchell, B. P. 1978. Spermatogenesis. In: C. A. Finn (Ed.) The Mammalian Testis. p 181. Cornell Univ. Press, Ithaca, NY.

Steel, R.G.D., and J. H. Torrie. 1980. Principles and Procedures of Statistics: A Biometrical Approach. (2nd Ed.). McGraw-Hill Book Co., New York.

Stewart, S. S., R. J. Emerick, and H. Kayongo-Male. 1993. Silicon-zinc interactions and potential roles for dietary zinc and copper in minimizing silica urolithiasis in rats. J. Anim. Sci. 71:946.