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Hydroxyurea exposure alters mouse testicular kinetics and sperm chromatin structure

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Abstract. The effects of hydroxyurea (HU) on testicular cell kinetics and sperm chromatin differentiation were investigated in mice. Whole testis, minced testicular cell suspensions and caudal epididymal sperm cells were obtained at 8 and 29 days after i.p. injections containing 0, 25, 50, 100, 200, 400 and 500 mg/kg HU × 5 days. Testis weights were unaffected by 25 mg/kg HU while 500 mg/kg caused up to a 50% loss of testicular weight by 29 days. Flow cytometrically measured acridine-orange (AO) stained testicular cells revealed altered population ratios at the highest dosages at 8 days and for all dosages except 25 mg/kg HU at 29 days. At 8 days, 400–500 mg/kg HU caused a near depletion of tetraploid cells. Flow cytometry of AO stained sperm, previously treated with acid to potentially induce DNA denaturation, was used to follow the shift from normal chromatin structure to an abnormal form with increased sensitivity to DNA denaturation *in situ*. The extent of DNA denaturation was quantitated for each cell by the computer-derived value alpha t, α_t = [red/(red+green) fluorescence]. The flow cytometry measures, standard deviation of α_t (SDα_t), mean of α_t (Xα_t) and cells outside the main peak of α_t (COMPα_t), gave similar dose response curves to the sperm head morphology assay. SDα_t was more sensitive than the Xα_t as a measure of HU-induced alteration of chromatin structure.

The major conclusions reached are that HU inhibits DNA synthesis, probably by inhibiting ribonucleotide reductase, causing maturation depletion of pachytene spermatocytes and, subsequently, depletion of meiotic daughter cells and differentiated cell types leading to mature sperm. This inhibition of DNA synthesis is related to an alteration of sperm chromatin structure and abnormal sperm head morphology.

Previous studies have shown that alkylating agents including ethylnitrosourea (ENU) (Evenson *et al.* 1985), thiotepa (Evenson *et al.* 1986a), and triethylenemelamine (TEM) (Evenson, Baer & Jost 1989), caused sperm chromatin structure abnormalities, defined as an increased susceptibility of nuclear DNA to denaturation *in situ* (Darzynkiewicz *et al.* 1975, Evenson, Darzynkiewicz & Melamed 1980, Evenson *et al.* 1985). The temporal and dose response curves for chromatin structure measures at this molecular level were highly correlated with gross sperm head morphology abnormalities. Current studies show that methyl methanesulphonate

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(MMS) (Evenson, Jost & Baer 1993) and ethyl methanesulfonate (EMS) (Evenson, Jost & Gandy 1993), two potent alkylating agents, also caused extensive chromatin structural abnormalities. However, in contrast with previously tested chemicals, 100% of the sperm had abnormal chromatin structure within 3 days of a single exposure, whereas sperm head morphology was not abnormal until 11 days after exposure. This can possibly be explained by the model of Sega & Owens (1983) that EMS and MMS alkylate free −SH groups on nuclear protamines in the late spermatid stages causing a destabilization of chromatin structure whereas ENU, thiotepa and TEM targeted early stages of spermatogenesis.

Agents other than alkylating agents have been shown to produce an abnormal chromatin structure as well as abnormal sperm head morphology. For example, the fungicide, methylbenzimidazol-2-yl carbamate (MBC) binds to tubulin, inhibiting polymerization and microtubule formation (Davidse & Flach 1977) and acts negatively on highly tubulin-dependent events of spermatogenesis (Carter & Laskey 1982, Evenson, Janca & Jost 1987) such as diakinesis through meiosis I and II. Furthermore, in the study by Evenson *et al.* (1987), sperm cells sampled 7 days post-treatment were exposed to MBC as early elongating spermatids and elongated spermatids, and demonstrated significant alterations of sperm chromatin structure and per cent abnormal sperm head morphology. Maturational changes occurring during this period, e.g. nuclear elongation and gross shaping of nuclear chromatin, could be related to tubulin-requiring events, and MBC inhibition of these events may have led to the alterations of chromatin structure and sperm head morphology that rendered the DNA more susceptible to acid-induced denaturation.

In our continuing efforts to understand mechanisms of induced sperm chromatin structure alterations, we have chosen to study the effects of a metabolic inhibitor, hydroxyurea (HU), which is known to inhibit cellular DNA synthesis.

The first unique step leading to DNA synthesis is the conversion of ribonucleotides to their corresponding deoxyribonucleotides, a reaction catalysed by ribonucleotide reductase (Lammers & Follman 1983). This rate-limiting enzyme in DNA synthesis consists of two non-identical subunits, M1 and M2, both of which are required for activity (Thelander, Eriksson & Akerman 1980, Thelander, Graslund & Thelander 1985). HU appears to work by destroying the tyrosine radical needed for enzyme action (Sjoberg *et al.* 1977) resulting in lowered pools of available DNA precursor d-GTP and d-ATP (Krakoff, Brown & Reichard 1968).

In contrast to the general assumption (Brachet 1985) that HU blocks cells at the G_1/S boundary, other studies suggest that cells enter the S phase at or about normal rates but are killed in early S phase. G_1 phase cells, not affected by HU exposure, then pass through the cycle more quickly whereas late S phase cells are delayed, thereby providing a partial synchronization of mitosis (Maurer-Schultze, Siebert & Bassukas 1988).

At high concentrations, HU is toxic to cells (Timson 1969) despite the rapid clearance time *in vivo*. The half-life for serum levels of HU after a 80 mg/kg dose was 5.5 h; after 24 h very little remained (Beckloff 1967). Concentration, duration of exposure, and sensitivity of organism affect the degree of antimitotic and cytotoxic effects (Timson 1975).

HU (3 mg/ml) in drinking water of mature male rats causes testicular atrophy and presumably inhibits mitosis of spermatogonia and synthesis of DNA by preleptotene spermatocytes (Setchell 1978). After treatment is stopped, the epithelium is repopulated from the stem spermatogonia, which remain unaffected (Mecklenburg *et al.* 1975).

HU decreased sperm motility by over 50% in mice in a dose responsive manner 35 days after injection (Ficsor & Ginsberg 1980). This was probably the result of non-hereditary disturbances during sperm development as HU is a weak mutagen and chromosome-breaking agent but a strong teratogen (Timson 1975). Significant decreases in testis weight and sperm

concentration were also noted in the Ficsor & Ginsberg (1980) study. These cells were late spermatogonial cells and primary spermatocytes when treated.

Wyrobek & Bruce (1975) have shown that HU produces sperm head abnormalities. Singh & Taylor (1981) concluded that there are species differences in magnitude of spermatogenic response to sperm production after treatment with HU and that the sperm morphology parameter is more reliable than sperm number or testis weight in determining mutagenic damage to the germinal epithelium for the *in vivo* mammalian system.

This study follows the effects of HU on testicular cell kinetics and differentiation of sperm nuclear chromatin as biological markers for reproductive toxin exposure.

METHODS AND MATERIALS

Animal care and chemical exposure

Male mice, 6–8-weeks-old, (C57B/6J × C3H/HeJ F_1) were received from Jackson Laboratories (Bar Harbor, ME, USA) and allowed to acclimatize in our animal facilities 7 weeks before chemical exposure. The mice were allowed free access to Purina Certified Rodent Laboratory Chow and deionized water, kept on a 07.00–19.00 h light schedule, and housed in polycarbonate cages with wire mesh tops and pine shavings for bedding.

At 13–15 weeks of age the mice were randomly placed in seven treatment groups of at least six mice each. The mice received 0.5 ml i.p. injections of phosphate-buffered saline (PBS; control) or PBS containing dosage levels of 25, 50, 100, 200, 400 and 500 mg/kg HU each day for 5 consecutive days.

Buffers and solutions

The following buffers and solutions were used:

TNE: 0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA (disodium); pH 7.4

HBSS: Hank's Balanced Salt Solution (Gibco)

Acridine orange (AO) buffer: 0.037 M citric acid, 0.126 M Na_2HPO, 0.001 M EDTA (disodium), 0.15 M NaCl; pH 6.0

AO stock solution: 1 mg AO/ml H_2O (chromatographically purified AO, Polysciences Inc., Warrington, PA, USA)

AO stain: 0.6 ml AO stock/100 ml AO buffer; final concentration of 6 μg AO/ml buffer

Acid-detergent solution: 0.08 N HCl, 0.15 M NaCl, 0.1% Triton-X 100; pH 1.4

Cell sample preparation

Three mice from each dosage were killed by cervical dislocation at 8 days. The remaining three mice were killed in the same manner at 29 days. Epididymi and testes were surgically removed and body and testes' weights recorded. All tissues were kept on ice (4°C) from the time of dissection to either measurement of fresh samples by flow cytometry (FCM) or freezing for later analysis.

Testicular cells.

Testes were placed in HBSS in 60 mm Petri dishes and minced with curved scissors. The cell suspension was placed in a 12 × 75 mm test tube and kept for several minutes to allow the tissue fragments to settle out. The cellular suspension was drawn off and filtered through a 53 μm Nitex filter mounted between a syringe barrel and the cap with its end cut off.

Epididymal sperm

The epididymi from each mouse were placed in TNE in a Petri dish and minced as for testes. After allowing the tissue fragments to settle out, the cells were filtered through a 153 μm Nitex syringe filter. The remaining sperm at 8 days were transferred to a −100°C freezer for storage. A single freezing and thawing of sperm has no known detrimental effect on their AO stainability as described (Evenson *et al.* 1989, Evenson & Thompson 1991). Twenty-nine day sperm were measured by FCM on the same day as sample preparation.

Two drops of filtered sperm were placed in each of two 12 × 75 mm tubes, stained with one drop of 0.5% aqueous Eosin Y for 30 min at room temperature, smeared onto glass slides and air dried. These were later scored for sperm head morphology by the method of Wyrobek & Bruce (1975).

Cell staining with AO

Aliquots (0.20 ml) containing 1–2 × 10^6 testicular cells or epididymal sperm/ml were mixed with 0.40 ml of the acid-detergent solution. Thirty seconds later, the cells were stained by adding 1.2 ml AO staining solution (Darzynkiewicz *et al.* 1976, Evenson *et al.* 1985). AO intercalated into native double-stranded DNA emits green fluorescence (F_{530}) when excited by blue (488 nm) laser light while AO associated with single-stranded nucleic acid (RNA or DNA) emits red fluorescence ($F\geqslant_{630}$) under the same excitation. The metachromatic fluorescence emitted from AO-stained testicular cells reflects the relative content of DNA (green fluorescence) and RNA (red fluorescence) (Darzynkiewicz 1979, Evenson & Melamed 1983, Evenson *et al.* 1986a,b).

Sperm Chromatin Structure Assay

When this AO staining technique has been used with sperm it has been termed the Sperm Chromatin Structure Assay (SCSA). Because normal, mature sperm cells contain virtually no RNA (Monesi 1965), very little red fluorescence is emitted (Evenson & Melamed 1983). Normal isolated sperm nuclei and/or whole cells treated with RNAse demonstrate the same fluorescence pattern which indicated that any red fluorescence observed is due to single-stranded (denatured) DNA (Evenson 1989, Evenson *et al.* 1985, 1989, Ballachey, Hohenboken & Evenson 1987). DNA in sperm with abnormal chromatin structure partially denatures under heat or low pH conditions yielding an increased red fluorescence (Evenson *et al.* 1980, 1985). Normal, native chromatin remains structurally sound with a minimum amount of red fluorescence emitted.

Defining alpha t (α_t)

Abnormal chromatin structure is defined as an increased susceptibility to acid- or heat-induced denaturation, and is determined by FCM 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, Darzynkiewicz & Kapuscinsky 1990), and is the ratio of red to total (red + green) fluorescence. Measurement of normal sperm produces a very narrow α_t distribution, while that of sperm with denatured DNA is usually broader with a higher mean channel and a larger per cent of cells outside the main population of α_t (COMPα_t). Standard deviation of α_t (SDα_t) describes the extent of chromatin structure abnormality within a population.

Flow cytometric measurements

Freshly prepared testicular and sperm samples from individual mice were stained with AO as described above and recorded FCM measurements of 5 × 10^3 cells per sample began 3 min after staining. Sperm samples collected at 8 days were frozen and later thawed in a 37°C water bath

before FCM measurement by the same method. The stained cells were measured in a Cytofluorograf II flow cytometer (Ortho Diagnostics, Inc., Westwood, MA, USA) equipped with ultrasense optics and a Lexel 100 mW argon ion laser operated at 35 mW with an excitation wavelength of 488 nm. Dual parameter FCM measurements were made to determine amounts of DNA *v.* RNA in testicular cells, and double-stranded *v.* single-stranded DNA in sperm cells. By use of dichroic mirrors and filters, fluorescent signals were separated into green (515–530 nm) and red (≥630 nm) components. The interfaced computer (2150 Data Handler, Ortho Diagnostic Systems, Inc.) calculated α_t values for each cell as well as α_t distribution values for each sample population both in live time and on retrieval from disk storage.

Statistical analysis

Data were analysed using the General Linear Model and correlation procedures of SAS-PC (SAS, 1988).

RESULTS

Weight data

As seen in Figure 1, body weights were not affected by any HU dosage at 8 or 29 days. Testes weights decreased significantly ($P<0.01$) for the two highest dosages (400–500 mg/kg) by 8 days. By day 29 testes' weights declined to about 50% of control after treatment with 500 mg/kg HU. The lowest dose of HU, 25 mg/kg, was the only dosage that did not affect testes' weight.

Testicular FCM data

Figure 2 is a dual parameter FCM cytogram showing raw data of measurements on AO stained testicular cells. Two tetraploid and diploid populations as well as three haploid populations are gated in the figure. The ratios of each cell type were determined by computer analysis. Note that these are relative ratios and, therefore, if one population decreases the relative percentage of another will increase whether or not an increase in the absolute number occurs. Figure 3 shows the changes in the ratios of testicular cell types in response to different exposure levels of HU measured at 8 and 29 days after exposure to 0–500 mg/kg HU. Eight days after exposure, the relative per cent total haploid cells increased slightly by exposure to 100–500 mg/kg HU ($P<0.01$). A concurrent decrease was seen in the per cent tetraploid population which nearly reached total depletion by exposure to 400–500 mg/kg. The per cent diploid cell population was not affected by any dosage. The three highest doses of HU (200–500 mg/kg) increased the elongated spermatid haploid cell subpopulation ($P<0.01$) while the round and elongating spermatids ratios did not change significantly.

By 29 days the testicular populations underwent dramatic changes in ratio of cell types (Figure 3). The per cent haploid cells steadily dropped to 50% of control for the highest dosage while relative diploid and tetraploid cell populations both increased by more than double. The relative ratios of tetraploid cells were significantly different ($P<0.05$) from control values at 50 mg/kg dosage, haploid cells at 100 mg/kg ($P<0.01$) and diploid cells at 200 mg/kg HU ($P<0.01$). At the same time the ratios of haploid subpopulations were altered as seen in the lower panels of Figure 3. All doses caused round spermatid ratios to be elevated with the maximum at 100 mg/kg HU (64% increase over the control). The ratios of elongating spermatids rose and were significantly different ($P<0.01$) from control at the three highest dosages. The elongated spermatids' ratio rapidly decreased from 32% of control and elongated spermatids were virtually eliminated by the 200 mg/kg dosage. All HU dosages caused significant decreases ($P<0.01$) in elongated spermatid numbers to elimination at 200 mg/kg.

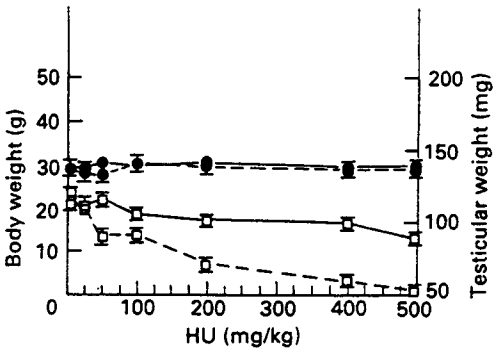

Figure 1. Least squares means of body (•) and testes' (□) weights and SE from three mice per dosage level of hydroxyurea (HU) at 8 (—) and 29 (---) days.

Two separate populations of both diploid and tetraploid cells can be distinguished by the AO staining method as gated in Figure 2. The percentage within each testis cell population was calculated as shown in Table 1. The most meaningful observation seen in Table 1 is the almost complete loss of tetraploid cell types at 8 days. Cells gated into box 1 are likely to be late pachytene spermatocytes while those in box 2 are early pachytene spermatocytes (Janca, Jost & Evenson 1986). Loss of late pachytenes began to occur with an exposure of 100 mg/kg while early pachytenes began to disappear by exposure to 50 mg/kg. A dosage of 400 mg/kg virtually eliminated all tetraploid cells at 8 days. After 29 days, there was a dramatic rebound in germ cell renewal with a relatively high per cent of tetraploid cells; this ratio was the highest in those mice that received the largest dosage and probably had the greatest damage to spermatogenesis. The ratios of diploid cells are shown but the interpretation is complicated by the presence of both germ and somatic cells.

Sperm FCM data

After 8 days, HU had no effect on the sperm population as measured by standard SCSA parameters, $X\alpha_t$, $SD\alpha_t$ and $COMP\alpha_t$, or as measured by the per cent abnormal sperm head morphology (ABN) (Figure 4). HU significantly affected sperm chromatin structure from mice killed at 29 days (Figures 4, 5). Mean α_t increased to significant levels ($P<0.01$) by 400 mg/kg. Note however, that $SD\alpha_t$ values are significantly different ($P<0.01$) from control starting at

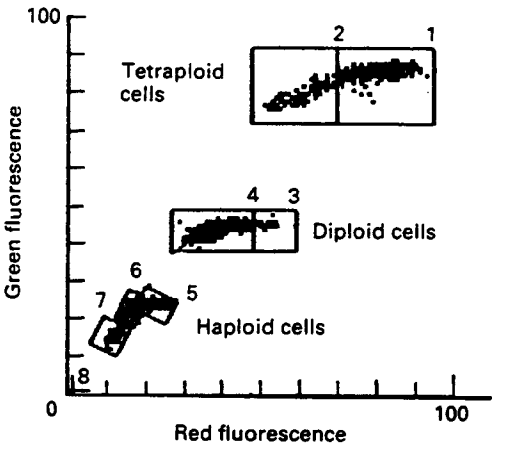

Figure 2. Dual parameter (green (DNA stainability) *v.* red (RNA content) fluorescence) cytogram of testicular cells gated into 7 populations. Sperm would appear just below the number 8.

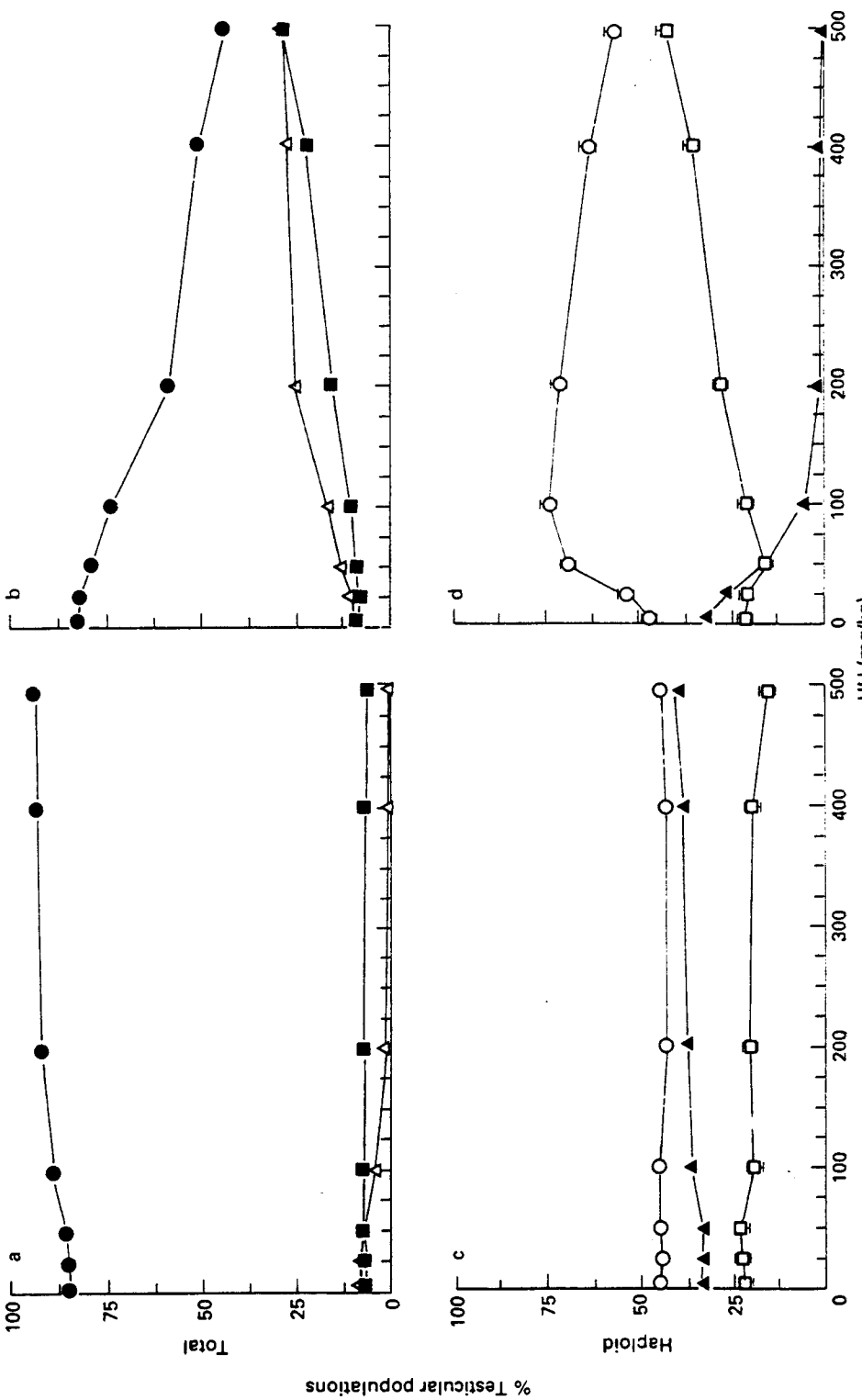

Figure 3. Percentages of testicular cells types—haploid (●), diploid (■), and tetraploid (△)—and percentage of haploid subpopulations—round (○), elongating (□) and elongated (▲) spermatids—found in mice **a,c** 8 and **b,d** 29 days after exposure to varying dosages of hydroxyurea (HU).

Table 1. Per cent diploid and tetraploid testis cell subpopulations as distinguished by the AO staining technique

	mg/kg HU						
	0	25	50	100	200	400	500
8 days							
Box 1 (%)	5.4	5.5	6.1	4.1	1.2	0.2	0.1
Box 2 (%)	3.3	3.3	1.9	1.0	0.6	0.2	0.3
Box 1/Box 2	1.6	1.7	3.2	4.1	2.0	1.0	0.3
Box 3 (%)	0.6	0.6	0.6	0.8	1.0	0.7	0.8
Box 4 (%)	6.2	6.0	6.6	6.4	6.3	5.4	5.6
Box 4/Box 3	10.3	10.0	11.0	8.0	6.3	7.7	7.0
29 days							
Box 1 (%)	5.5	7.3	8.7	10.8	17.0	18.1	15.1
Box 2 (%)	3.7	3.6	3.8	5.6	9.7	9.0	12.8
Box 1/Box 2	1.5	2.0	2.3	1.9	1.8	2.0	1.2
Box 3 (%)	0.9	0.9	1.1	1.4	3.1	4.7	7.8
Box 4 (%)	7.1	7.5	7.5	9.2	12.1	19.1	23.1
Box 4/Box 3	7.9	8.3	6.8	6.6	3.9	4.1	3.0

100 mg/kg HU. Increased COMPα_t is significant for the three highest dosages (200–500 mg/kg). The greatest response for FCM sperm parameters occurred at 400 mg/kg HU. Pertinent correlation coefficients are given in Table 2. Regression analysis was performed on SDα_t and ABN dose-response lines. Linear regression revealed slopes of the two lines are not different ($P>0.05$) at 8 days. The same was true for quadratic regression analysis after 29 days.

Intact sperm and sperm with detached heads and tails are readily distinguishable using AO staining in conjunction with green peak *v.* area fluorescence signal processing. An interesting dose-response curve showing the per cent detached heads *v.* dosage is shown in Figure 6. The greatest effect, per cent free heads and per cent ABN, occurred at 400 mg/kg HU.

DISCUSSION

This study supports the view that HU interferes with testicular cell DNA synthesis, probably by inhibition of ribonucleotide reductase, leading quickly to a reduction in the normal pool size of pachytene spermatocytes. This action thus leads to a depletion of meiotic daughter cells and a disruption in the flow of spermiogenesis. HU does not kill the spermatogonia stem cell which, following clearance of the HU, repopulates the germ cells with normal differentiation kinetics.

The most dramatic occurrence, 8 days following exposure, was the near total disappearance of tetraploid cell types. Thus, DNA synthesis was probably inhibited in type B spermatogonia. Passage from type B spermatogonia (diploid) to leptotene spermatocytes (tetraploid) takes 2.86 days (Oakberg 1956a,b). Thus, DNA synthesis was probably blocked or dramatically inhibited by the HU and S phase cells were perhaps killed which would deplete the tetraploid population. Other than the tetraploid population depletion, the relative ratios of other populations remained nearly the same suggesting that cellular differentiation proceeded on a near normal schedule following HU exposure.

By day 29, a dramatic shift to a lower relative ratio of round spermatids was observed. This lower ratio is in part due to the very significant increase in tetraploid and diploid cells present as part of a new wave of spermatogenesis following exposure, maturation depletion and

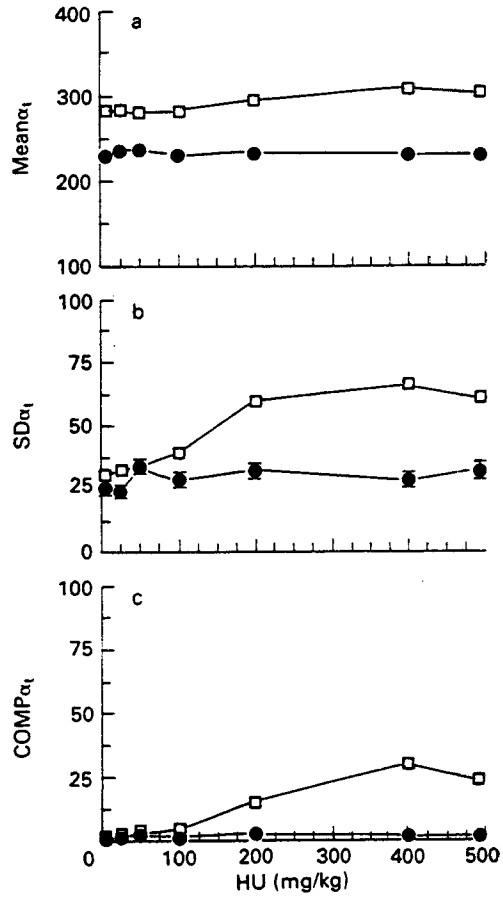

Figure 4. Dose response curves of a $X\alpha_t$, b $SD\alpha_t$ and c $COMP\alpha_t$ at 8 (•) and 29 (□) days after exposure to hydroxurea (HU) dosages shown.

possible kill of S phase cells. The higher the concentration of HU, the greater the cell block and possible kill, and thus the higher the relative cell types that are present from the rebound effect. Testes' weights were only 50% of normal at 29 days. The pattern seen at 29 days has kinetic characteristics previously seen in pubertal mice (Janca *et al.* 1986) with the appearance of the first wave of cells in spermatogenesis. Likewise, at 29 days, an increase of round and elongating spermatids was seen. Twenty-six days is the time lag between type A spermatogonia and the appearance of elongated spermatids (Figure 7). Thus, appearance of elongated

Table 2. Correlation coefficients of dosage of hydroxyurea with SCSA parameters

	$X\alpha_t$	$SD\alpha_t$	$COMP\alpha_t$	ABN (%)
8 days ($n = 21$)				
Dose	−0.18	0.26	0.05	−0.25
ABN (%)	0.24	0.11	0.18	—
29 days ($n = 21$)				
Dose	0.75**	0.89**	0.92**	0.82**
ABN (%)	0.75**	0.92**	0.93**	

ABN, abnormal sperm head morphology.

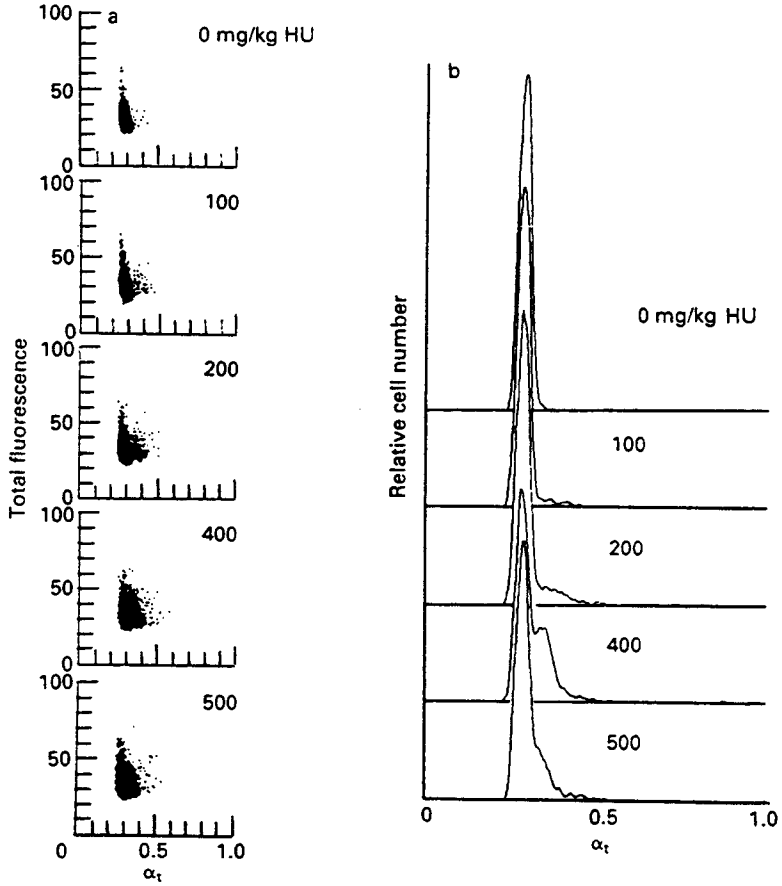

Figure 5. Representative **a** FCM cytograms (total fluorescence *v.* α_t) and corresponding **b** α_t histograms of cauda sperm isolated at 29 days after mice were treated with 0, 100, 200, 400 and 500 mg/kg hydroxyurea (HU).

spermatids may have been expected at this time, but the trauma of the HU exposure may have delayed the spermatogenic process by several days.

Eight days after exposure, cauda sperm showed no increased susceptibility to DNA denaturation *in situ* indicating normal chromatin structure. Likewise, sperm head morphology was normal. These sperm were elongated spermatids at the end of exposure and elongating

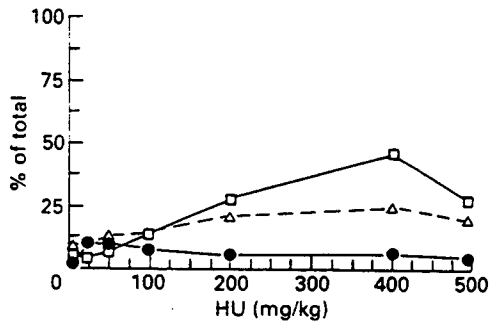

Figure 6. Percentage abnormal sperm head morphology at 8 days (●) and 29 days (□) and percentage sperm with detached heads at 29 days (△) over the range of 0–500 mg/kg hydroxurea (HU).

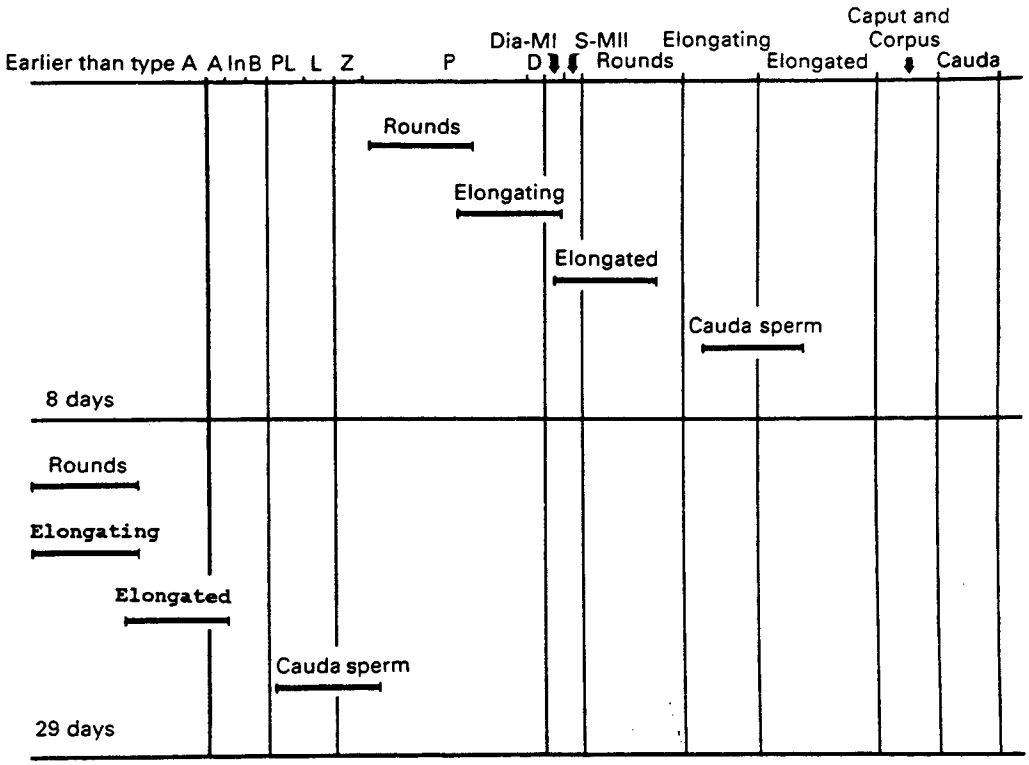

Figure 7. Diagram representing the spermatogonial stages during the 5 day hydroxyurea (HU) exposure period, which give rise to round, elongating and elongated spermatids and cauda spermatozoa 8 and 29 days after HU exposure. Bars show stages during which cells were treated. Spermatogonial stages: type A spermatogonia (A), intermediate spermatogonia (In), type B spermatogonia (B). Meiotic stages: preleptotene (PL), leptotene (L), zygotene (Z), pachytene (P), diplotene (D), diakinesis-reductional division (Dia-MI), secondary spermatocyte-equational division (S-MII). Spermatid stages: round, elongating and elongated spermatids. Duration of the spermatogenic stages is based on Oakberg (1956a,b) with the total spermatogenic cycle at 34.5 days: timing of sperm movement through epididymis is from Sega (1974). The figure is drawn proportionately on a linear scale.

spermatids at the beginning of exposure (Figure 7). Thus, HU had no effect on late maturation of sperm cells, and its effects are found earlier when germ cells are undergoing DNA synthesis.

In contrast, at 29 days, the cauda had up to about 25% sperm with abnormal chromatin structure (COMPα_t). These cells were in pachytene stage at the beginning of exposure and approximately leptotene stages at the end of exposure (Figure 7). As only 25% of the cells were affected, a stage-specific effect is likely. Note that the $\bar{x}$ α_t values were relatively constant, but the SDα_t had a significant increase. An increased SDα_t may reflect a spectrum of abnormalities in the entire population including those in the main population. This pattern is similar to that seen with exposure to alkylating agents such as ethylnitrosourea (Evenson *et al.* 1985) which have an effect on early spermatogenesis stages. Of interest, increasing concentrations of HU caused an increased per cent of detached heads by 29 days. Thus, HU caused multiple effects on differentiation which may be related or separate phenomena.

The most important observation in this study is that an agent which interferes with DNA synthesis also causes abnormal sperm chromatin structure, probably related to the observed increase of sperm heads with abnormal morphology. It is suggested from other studies on cattle

(Ballachey *et al.* 1987) and humans (Evenson 1989) that these sperm are infertile. Ward & Coffey (1991) have speculated that sperm must have a proper chromatin organization in order to function properly for fertilization and early embryo development. The mechanism of interrupted DNA synthesis leading to altered chromatin structure is not known, but continues to be investigated.

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