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• *Original Contributions*

EFFECTS OF ACETAMINOPHEN AND HYDROXYUREA ON SPERMATOGENESIS AND SPERM CHROMATIN STRUCTURE IN LABORATORY MICE

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Abstract — High doses of acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg) given intraperitoneally daily for 5 d caused reduction in relative testicular weight in mice (B6C3/F1/BOM M). Testicular atrophy of several tubules was seen in the hydoxyurea-treated mice 5 d after the last exposure, whereas acetaminophen did not lead to such changes. Exposure to acetaminophen caused neither a depletion of glutathione in the testis nor a marked increase in covalent binding. In contrast, significant decreases in the incorporation of thymidine into the testis were observed during the first 3 h following a single treatment with acetaminophen (100 to 400 mg/kg) or hydroxyurea (100 to 200 mg/kg). In mice treated with acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg) daily for 5 d, flow cytometric analysis revealed large reductions in one of the tetraploid populations of testicular cells (mostly early pachytene spermatocytes) on days 5 and 10. Changes in the populations of the various spermatid stages occurred later; thus, both compounds appeared to cause a delay in spermiogenesis. Indications of abnormal chromatin structure were seen in an increased frequency of vas deferens sperm on days 27 and 33 after the last exposure, when measured as increased susceptibility towards DNA denaturation in situ. In conclusion, high doses of acetaminophen or hydroxyurea inhibit DNA synthesis in the testis. The present data indicate that this leads to reduced testicular weight, a reduction in the number of early pachytene spermatocytes, changes in the proportions of the various spermatid stages, and an apparent alteration in sperm chromatin structure.

Key Words: impaired spermatogenesis; inhibited DNA synthesis; sperm chromatin structure.

INTRODUCTION

Acetaminophen is widely used as a nonprescription analgesic and antipyretic. Overdosing with acetaminophen can cause hepatic necrosis in both humans and laboratory animals (1–3), and prolonged human use has been implicated in chronic renal disease (4). Moreover, genotoxic effects of acetaminophen have been observed. In vivo, acetaminophen has been shown to cause chromosome aberrations in bone marrow cells from exposed mice (5–7). In human volunteers given therapeutic doses of acetaminophen, chromosomal effects were oberved in studies on Czech and Norwegian populations. Increased frequencies of sister-chromatid exchanges (SCE) and chromatid breaks in lymphocytes (8,9), and micronuclei in buccal mucosa cells (10) were observed. However, no increase in chromosomal aberrations in human lymphocytes were reported in a study on British volunteers (11).

The liver necrogenic effects of acetaminophen are considered to be caused by reactive metabolite(s) that oxidize and/or covalently bind to cellular proteins (12,13). Reactive acetaminophen metabolites may also explain some of the genotoxic effects observed after acetaminophen exposure (14–16). In addition, unmetabolized acetaminophen may cause

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genotoxic effects by inhibiting DNA synthesis (17). Studies utilizing cell lines with different levels of ribonucleotide reductase showed that acetaminophen reduced DNA synthesis by a specific inhibition of ribonucleotide reductase. Further studies with these cell lines showed that acetaminophen-induced sister chromatid exchanges and chromosome aberrations were correlated with the reduced DNA synthesis (18).

Placke and co-workers (19) demonstrated that other tissues in addition to liver and kidney are damaged after exposure to high doses of acetaminophen. They reported degenerative and necrotic changes in lung, testis, and lymphoid tissue of mice. High doses of acetaminophen in rats have also been reported to lead to testicular atrophy and impaired spermatogenesis (20,21). Acetaminophen-induced pancreatitis has also been observed (22,23). Recently, Evenson and Jost (24) reported that hydroxyurea, another inhibitor of ribonucleotide reductase (25), altered cell proliferation and differentiation of mouse testicular cells. In the present study we examined whether reactive acetaminophen metabolites are formed in testicular cells and if acetaminophen interferes with DNA synthesis. Furthermore, we have studied the effect of acetaminophen on spermatogenesis and on the structure of sperm chromatin using flow cytometry. Changes were compared with the effects obtained after hydroxyurea treatment.

MATERIALS AND METHODS

Chemicals

Acetaminophen was purchased from Riedel–deHaën AG, Hannover, Germany; [methyl-^{3}H] thymidine ([^{3}H]TdR; 20 Ci/mmol), and [^{3}H] acetaminophen (1.40 Ci/mmol) from NEN Dupont Dreiech, Germany; hydroxyurea from Sigma Chemical Company, St. Louis, MO; and acridine orange (AO, chromatographically purified) from Polysciences, Wartburg, Germany.

Animals

Male mice used in this study (B6C3/F1/BOM M, 6 to 8 weeks old, 23 to 26 g at the start of the experiments) were housed in plastic cages on hardwood bedding. They were given standard pelleted feed (Ewos R3, Astra Ewos AB, Södertälje, Sweden) and water ad libitum. The animals were weighed at the initiation of the study and immediately prior to sacrifice.

Histology of the testis and liver

Five mice in each group were given i.p. injections with either acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg) for 5 consecutive days. On days 5 and 10 after last treatment, the liver and testis with caput epididymis were fixed in 2% neutral glutaraldehyde, sectioned and stained with hematoxylin–eosin. All organs were examined microscopically for changes related to chemical exposure. An ocular scale was used to measure the diameters of round/oval seminiferous tubules with normal appearance. Twenty tubules per testis were measured for two control, two acetaminophen-, and three hydroxyurea-treated mice.

Covalent binding of acetaminophen and nonprotein sulphydryls levels

Five mice in each group were given single i.p. injections of [^{3}H]-acetaminophen (600 mg/kg; 500 dpm/nmol) in phosphate-buffered saline (PBS) pH 7.4. After various time intervals the mice were killed by cervical dislocation. Testis and kidneys were excised and homogenized in 4 vol ice-cold PBS. Covalent binding to protein was examined by transferring 2×50 μL aliquots of the homogenate to Whatman GF/C glass filters (Maidstone, UK). Macromolecules were precipitated on the filters by immersing them in 95% ethanol. The filters were washed several times in organic solvents and covalent binding was measured by liquid scintillation counting (26).

GSH levels were estimated using the method of Ellmann (27) to measure nonprotein sulphydryls. The homogenate was precipitated with the same volume of 4% sulfosalicylic acid and centrifuged. The supernatant (0.2 mL) was added to 1.8 mL 0.1 mM 5,5-dithiobis-(2-nitrobenzoic acid) prepared in 0.1 M Na-phosphate buffer (pH 8.0). The amount of nonprotein sulfhydryl was calculated from the absorbance at 412 nm, with GSH as standard.

Incorporation of [methyl-^{3}H]thymidine ([^{3}H]TdR)

Five mice in each group were given i.p. injections of acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg) in PBS (injection volume around 600 μL) daily for 5 d (except where another treatment is indicated). Control animals were given the corresponding volume of PBS. After various time intervals [^{3}H]TdR (3 μCi/g mouse in PBS) was injected i.p. in treated and control animals. One hour after TdR injection, the mice were killed by cervical dislocation. Testes were removed, weighed, and put into 2 mL of saline on ice. The samples were then homogenized in a Potter–Elvehjem homogenizer, after which DNA was extracted 3 times with ice-cold 5% trichloroacetic acid (TCA) at 90 °C. Aliquots of the DNA samples were counted in a Packard scintilla-

tion counter, and DNA was measured according to Burton (28). The incorporation of [^{3}H]TdR was calculated as counts/μg DNA.

Testicular germ cells

In experiments concerning possible effects of treatment on testis weights, composition of testicular cells, and sperm chromatin structure, animals were dosed daily for 5 d. Body weights were recorded on the first day of treatment and immediately after cervical dislocation. Testicular cells were prepared as previously described (29). In short, both testes were surgically removed, weighed, and minced with curved scissors into a cellular suspension in a 60 mm petri dish containing 2 mL Hank's buffered-saline solution at 4 °C, and then transferred to 12 × 75 mm polystyrene tubes. After settling of tissue fragments, the supernatants were filtered through 53 μm nylon mesh (Tetko, Inc., New York, NY) into 12 × 75 mm tubes, and kept on crushed ice (4 °C) until stained and measured by flow cytometry.

Vas deferens sperm

Vas deferens were surgically removed and placed in a 60 mm petri dish containing 2.0 mL TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA (disodium) pH 7.4) at 4 °C. The sperm were squeezed out with a blunt instrument into the medium, aspirated, and expelled several times through Pasteur pipettes, then filtered through 153 μm nylon mesh into 12 × 75 mm tubes, and kept on crushed ice until measured by flow cytometry (FCM) according to Evenson et al. (29).

Cell staining with acridine orange

Two hundred microliter aliquots containing 1 to 2 × 10^6 testicular cells or vas deferens sperm per mL were mixed with 400 μL 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.4. After 30 sec, the cells were stained by adding 1.2 mL of a solution containing 6 μg chromatographically purified acridine orange (Polysciences Inc., Warrington, PA) per mL of AO buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0; [30,31]).

When excited by blue laser light, AO intercalated into native, double-stranded DNA fluoresces green; AO associated with single-stranded nucleic acid (DNA or RNA) emits red fluorescence. The metachromatic fluorescence observed in AO-stained testicular cells reflects the relative content of DNA (green fluorescence) and RNA (red fluorescence) (30,32).

Sperm chromatin structure assay

To distinguish it from other acridine orange staining protocols, the staining procedure described above has been termed the sperm chromatin structure asay (SCSA) when utilized with sperm. Although this low pH treatment does not cause denaturation of histone-complexed DNA, it apparently causes partial DNA denaturation in sperm with altered chromatin structure (31). Abnormal chromatin structure, defined as an increased susceptibility to acid-induced denaturation, 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) (30), 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 broader and has a larger percent of cells outside the main population of α_t (COMP α_t or percent abnormal cells).

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 3.0 min after staining. Sperm samples collected on the various sampling days were frozen until 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) equipped with a Coherent 5W argon ion laser operated at 250 mW with an excitation wavelength of 488 nm. Dual parameter FCM measurements were made to determine amounts of (a) DNA vs. RNA in testicular cells and (b) double-stranded vs. single-stranded DNA in sperm cells. By use of dichroic mirrors and filters, fluorescence signals were separated into green (515 to 530 nm) and red (> 630 nm) components. The interfaced computer calculated α_t values for each cell as well as α_t distribution values for each sample population both in live time and upon retrieval from disk storage. The percentage of cells with increased α_t values are equivalent to the percentage of cells described here with abnormal chromatin structure. The samples of testicular cells from days 5 and 10 were analyzed on an Argus 100 flow cytometer (Skatron, Tranby, Norway).

Statistical analysis

All statistical analysis were performed using Student's t test ($P < 0.05$).

RESULTS

Body and relative testis weights and mortality

During the 5-d treatment period, the mice that received 400 mg/kg acetaminophen displayed a loss of body weight compared to initial weight. During the first 5 d after the last treatment, there was no net body weight gain in the acetaminophen- or hydroxyurea-treated mice, whereas control animals gained 1.3 g (4%). Throughout the remainder of the posttreatment period there were no differences between the controls and the treated animals (Figure 1). During the treatment period, the exposed mice showed signs of weakness. This was temporary and lasted 1 to 2 d after treatment. The mortality for the three groups (n = 24 to 26) were 2, 4, and 1, respectively, in the control, acetaminophen, and hydroxyurea treatments. Mortality appeared to be due to fighting.

In control animals, the mean testis weights varied from 191 to 231 mg on days 27 and 33. Treatment led to reductions in both the absolute and relative testis weights. Hydroxyurea caused the greatest reductions in testes weights. On days 27 and 33 after treatment, the testis weights were approximately 40 to 45% lower than concomitant control values (Table 1). Acetaminophen-treated mice had testes weights that were approximately 16 to 18% lower than the controls. By day 45, the relative testes weights returned to control values (data not shown). The 500 mg/kg acetaminophen treatment for 5 d was included in the study to examine the effects of a dose that is closer to the hepatotoxic dose in mice (600 mg/kg, unpublished). This treatment led to an even greater reduction of testis weights than the 400 mg/kg dose on day 33.

Histology

As can be seen in Figure 2, the testes of the acetaminophen-treated animals were not discernably different from control testes on day 5 after

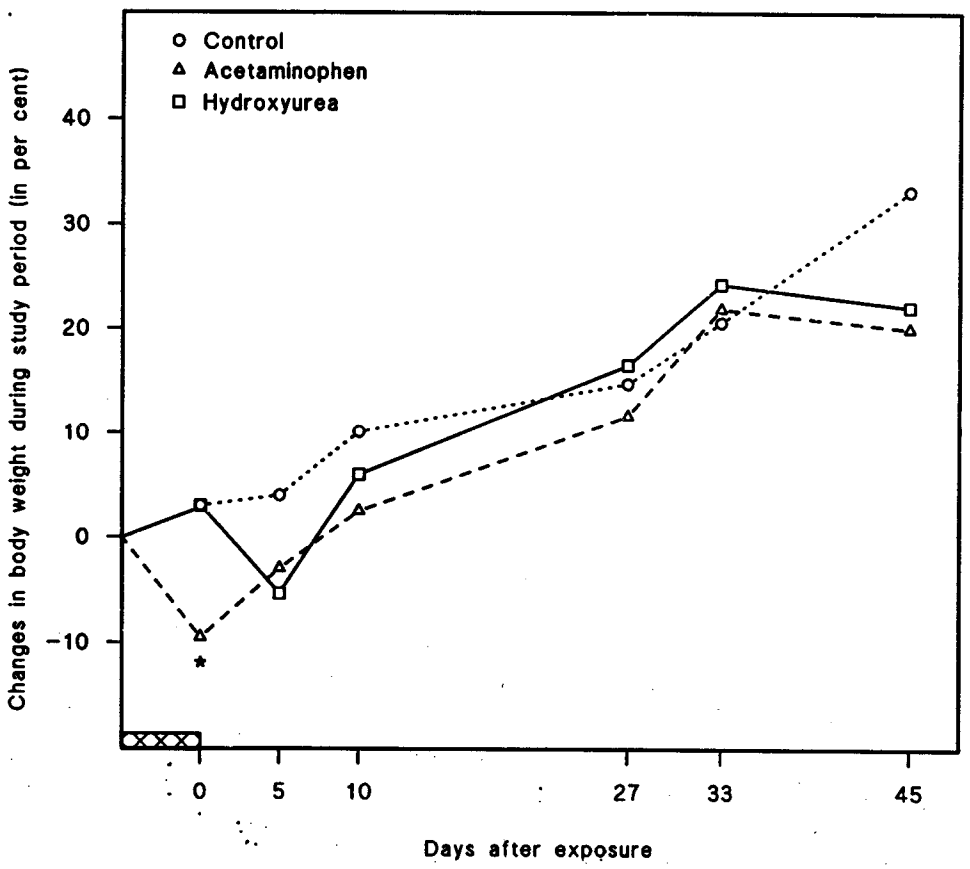

Fig. 1. Growth curves for mice given five daily doses of either acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg). Growth is expressed as percent change from the initial body weights. Each point is the mean value for three to five animals per group with the exception of day 33 controls, which consisted of two animals. *Significantly different ($P < 0.05$) from the control group by Student's t test.

Table 1. The tests weights (mg) (mean ± SE) of mice on days 27 and 33 after treatment with hydroxyurea or acetaminophen

Days	Control	Hydroxyurea (200 mg/kg)	Acetaminophen (400 mg/kg)	Acetaminophen (500 mg/kg)
27	191 ± 2.7 (3)	116 ± 8.5[a] (4)	160 ± 4.2[a,b] (4)	ND
33	231[c] (2)	127 ± 12.2[a] (4)	189 ± 9.0[a,b] (4)	138 ± 6.7[a,d] (4)

ND = no data.
[a]Significantly different ($P < 0.05$) from the controls by Student's t test.
[b]Significant difference between acetaminophen and hydroxyurea ($P < 0.05$).
[c]$Mean_1 = 222$; $mean_2 = 240$.
[d]Significant difference between acetaminophen (400 mg/kg) and acetaminophen (500 mg/kg) ($P < 0.05$).

last exposure. Hydroxyurea treatment, on the other hand, led to disruption of some of the seminiferous tubules. There were a number of atrophied tubules surrounded by normal tubules. The atrophied tubules, which contained large vacuoles and few cells, had considerably smaller diameters than normal tubules. A more subtle change related to treatment was observed when the diameters of apparently normal seminiferous tubules having round/oval cross-sections were measured. The diameters of the seminif-

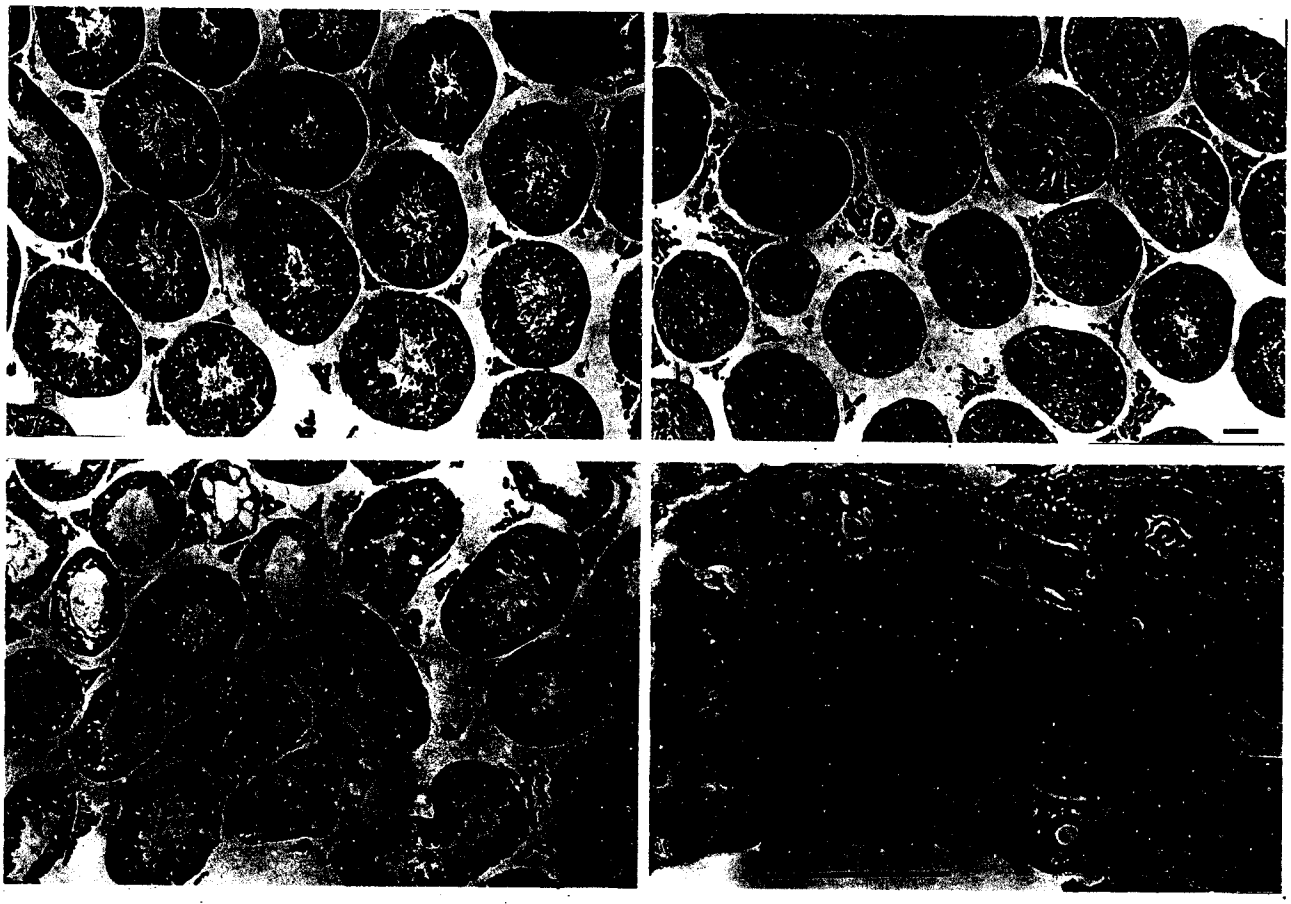

Fig. 2. Effects of five consecutive daily doses i.p. of acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg) on the testes of mice on day 10 after the last dose. The testes of (a) control, (b) acetaminophen-treated animal revealing normal normal seminiferous tubules, (c) hydroxyurea-treated aminal with several atrophic tubules, and (d) an atrophic tubule from a hydroxyurea-treated animal containing Sertoli cells only (arrows). Scale bar, 50 μm.

erous tubules of treated mice on day 5 were smaller than those of controls. Compared to control values, the mean percentages ± SE were as follows: controls 100 ± 1.5%, acetaminophen 94.0 ± 1.6%, hydroxyurea 91.4 ± 1.3%. The differences were statistically significant ($P \leq 0.003$; Student's t test). In addition to these changes, the hydroxyurea-treated mice had many large nonsperm cells in the tubules of the caput epididymis. Such cells were only seldomly seen in the control or acetaminophen-treated mice (data not shown).

On day 10 after the last exposure, half of the mice in the hydroxyurea group had areas of atrophy in the testis. In some instances the tubules were virtually empty and some were collapsed. A majority of the atrophied tubules appeared to contain mostly Sertoli cells (Figure 2). The testes of acetaminophen-treated mice were not discernably different from the controls.

The livers of the acetaminophen-treated mice on days 5 and 10 appeared to be histologically normal, whereas the livers of the mice treated with 200 mg/kg hydroxyurea were characterized by extensive intracellular vacuolization. Vacuolization was seen throughout the liver and was not confined to specific regions. The liver sections were stained using a periodic acid Schiff procedure, and the vacuoles did not stain positively for glycogen.

Covalent binding and depletion of nonprotein sulphydryls

Hepatotoxicity of acetaminophen correlates with the formation of reactive metabolites that deplete glutathione, which accounts for a great proportion of nonprotein sulphydryls, and bind covalently to cellular macromolecules (3). Thus, the possible formation of reactive acetaminophen metabolites in the testes was studied by measuring the level of nonprotein sulphydryls and radiolabeled acetaminophen covalently bound to macromolecules. Administration of 600 mg acetaminophen/kg to mice 2 h before sacrifice caused a marked reduction in liver glutathione, whereas no effect was observed in testicular cells (Table 2). The covalent binding of radiolabeled acetaminophen in the liver was one order of magnitude higher in the liver than observed in the testis (Table 3).

Table 2. The effects of acetaminophen on nonprotein sulphydryl levels reflecting glutathione (GSH) concentrations

Treatment	Nonprotein sulphydryl levels (nmol/mg protein)	
	Liver	Testis
Control	46.6 ± 9.1	72.6 ± 4.0
Acetaminophen	11.8 ± 5.7	79.8 ± 5.9

Mice were given 600 mg acetaminophen/kg 2 h before the animals were killed. Values are the means ± SD of 4 or 5 animals.

Table 3. Covalent binding of [^{3}H]acetaminophen

Organ	Covalent binding (pmol/mg protein)
Liver	1002 ± 420
Testis	115 ± 26

Mice were given 600 mg [^{3}H]acetaminophen 2 h before the animals were killed. Values are means ± SD of 4 or 5 animals.

DNA synthesis

The recent reports concerning acetaminophen's inhibitory effects on thymidine incorporation of proliferating cells both in vivo and in vitro (16,18) caused us to suspect that it might also affect spermatogenesis in vivo. Intraperitoneal injections (five daily doses) with either acetaminophen or hydroxyurea significantly reduced the incorporation of radiolabelled thymidine in a dose-related manner (Figure 3). Radiolabelled thymidine was incorporated during the period 15 to 75 min following the last injection

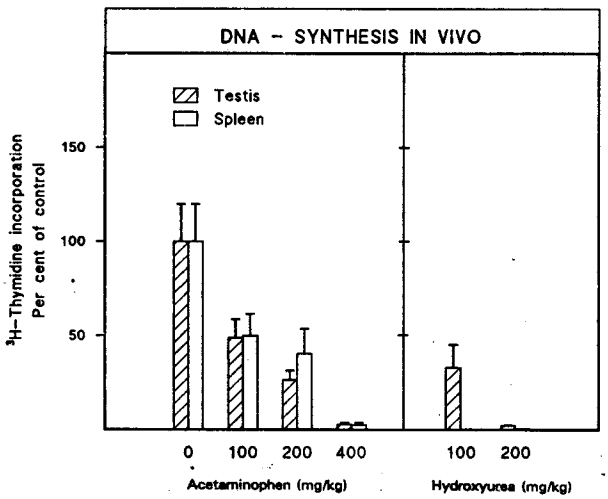

Fig. 3. The effects of treatment with various doses of acetaminophen or hydroxyurea on the in vivo incorporation of ^{3}H-thymidine into testicular cells. ^{3}H-thymidine was incorporated from 15 to 75 min after i.p. treatment with the test compounds. The 100% control values for ^{3}H-thymidine incorporation into testis and spleen are equal to 129 and 695 dpm/μg DNA, respectively. Bars = SE.

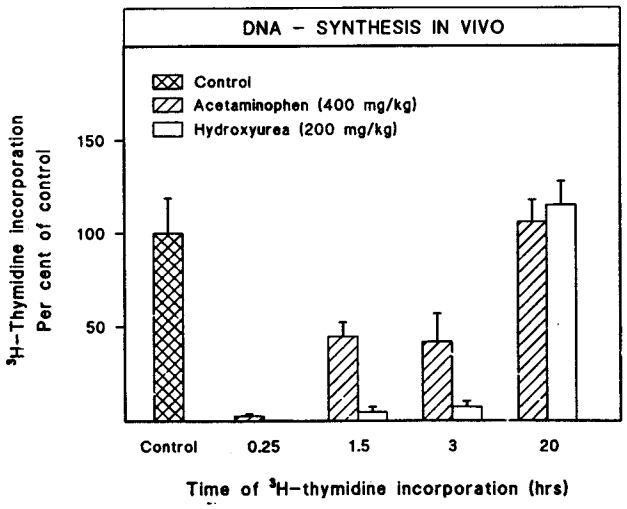

Fig. 4. The effects of acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg) on the in vivo incorporation (60 min) of ^{3}H-thymidine into testicular cells at various time periods following treatment. The 100% control value for ^{3}H-thymidine incorporation into testis is equal to 136 dpm/μg DNA. Bars = SE.

of the test substance. For the lowest concentrations tested (100 mg/kg), acetaminophen and hydroxyurea caused approximately 50 and 65% reductions, respectively, compared to control values. At the highest doses, the incorporation of thymidine was nearly totally blocked. The relative inhibition of thymidine incorporation into testis and spleen were similar for acetaminophen-treated mice, whereas hydroxyurea caused even greater inhibition in the spleen than in the testes.

As can be seen in Figure 4, incorporation of thymidine into the testis during the first hour after a single i.p. injection was effectively blocked following treatment with equimolar doses of acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg). Four hours following treatment, thymidine incorporation into the testes of acetaminophen-treated animals was still only 45%, whereas in hydroxyurea-treated mice it was 8% of control values. After 20 h the incorporation rate had returned to approximately normal levels, or perhaps was slightly higher than in controls.

Composition of testicular cells. Flow cytometry has been shown to rapidly provide information as to whether or not a chemical exposure may cause selective cell death or alter the kinetics of cell proliferation and differentiation of testicular cells. As shown in Figure 5, dual parameter green (DNA) vs. red (RNA) fluorescence FCM measurements of AO-stained mouse testicular cells resolved cell populations with tetraploid, diploid, and haploid DNA content; mouse diploid thymocytes were used as a ploidy reference marker. Two subpopulations of both tetraploid and diploid cells, differing in red fluorescence (RNA), and three subpopulations of haploid cells, differing in red (RNA) and green (DNA stainability) flourescence, were discernible. The ratios of each cell type were determined by computer analysis; it is noted that these are relative ratios and,

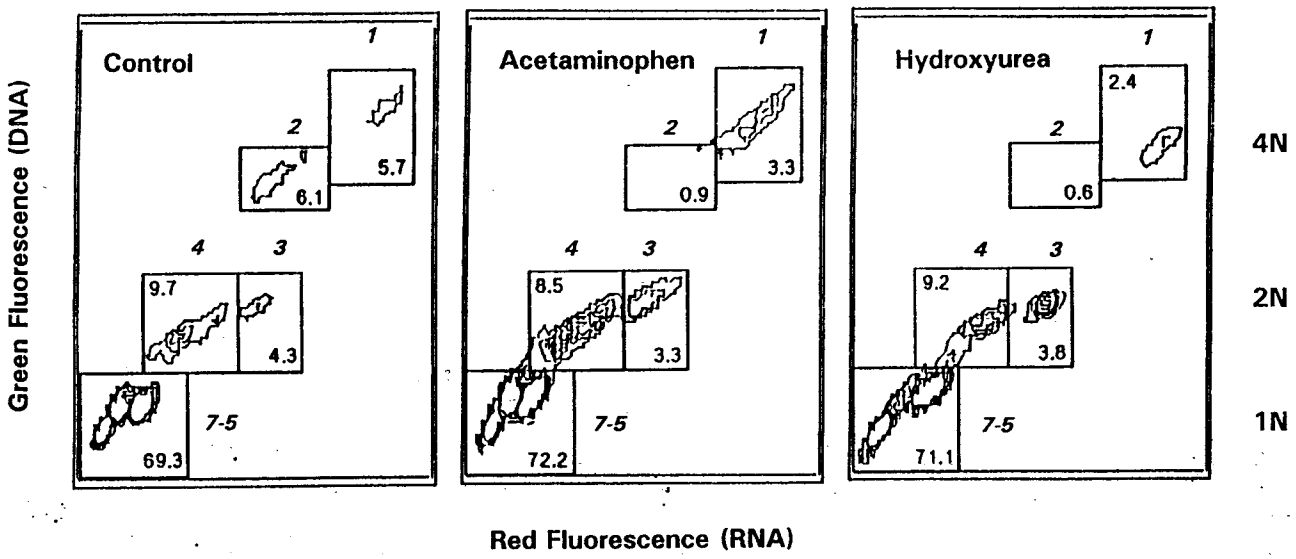

Fig. 5. Flow cytometer scatterplots showing effects of five daily doses with either acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg) on dual parameter (DNA vs. RNA) distribution of acridine orange-stained testicular cells obtained from mice on day 5 after last exposure. Testicular cells were gated into seven populations (boxes), and the numbers within each box represents the percentage of cells in that box.

therefore, if one population decreases, the relative percentage of another will increase whether or not an increase in the absolute number occurs.

Changes in the ratios of testicular cell types in response to different exposure levels of hydroxyurea or acetaminophen measured 26 d after last exposure are shown in Figure 6. With increasing doses of hydroxyurea there were trends whereby the proportion of haploid cells decreased while both the diploid and tetraploid populations increased. The effects of acetaminophen on testicular cell kinetics were much less obvious than for hydroxyurea. Only the highest dose of acetaminophen (400 mg/kg) caused alterations in the proportions of the haploid populations that could still be seen after 26 d. The trends were similar to those observed in the hydroxyurea-treated mice with respect to the haploid populations.

The data in Figure 6 show that only minor variations could be seen in the composition of testicular cells on the last day of treatment (day 0). However, 5 and 10 d after last exposure with acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg) marked alterations in the ratios of testicular cell types could be seen (Figure 7). In the samples from both 5 and 10 d after treatment there were statistically significant reductions in the tetraploid populations compared to controls (Table 4). Two separate populations of both diploid and tetraploid cells can be distinguished by the AO staining method, as shown in Figure 5. The percentage within each testis cell population was calculated, as shown in Figure 5. The most striking observation in Table 4 is the marked decrease of the tetraploid cell types at 5 and 10 d. Cells gated into box 1 are likely late pachytene spermatocytes, while those in box 2 are likely early pachytene spermatocytes and, to a certain degree, also type B spermatogonia (34). The greatest reductions occurred in the early pachytene spermatocyte fraction

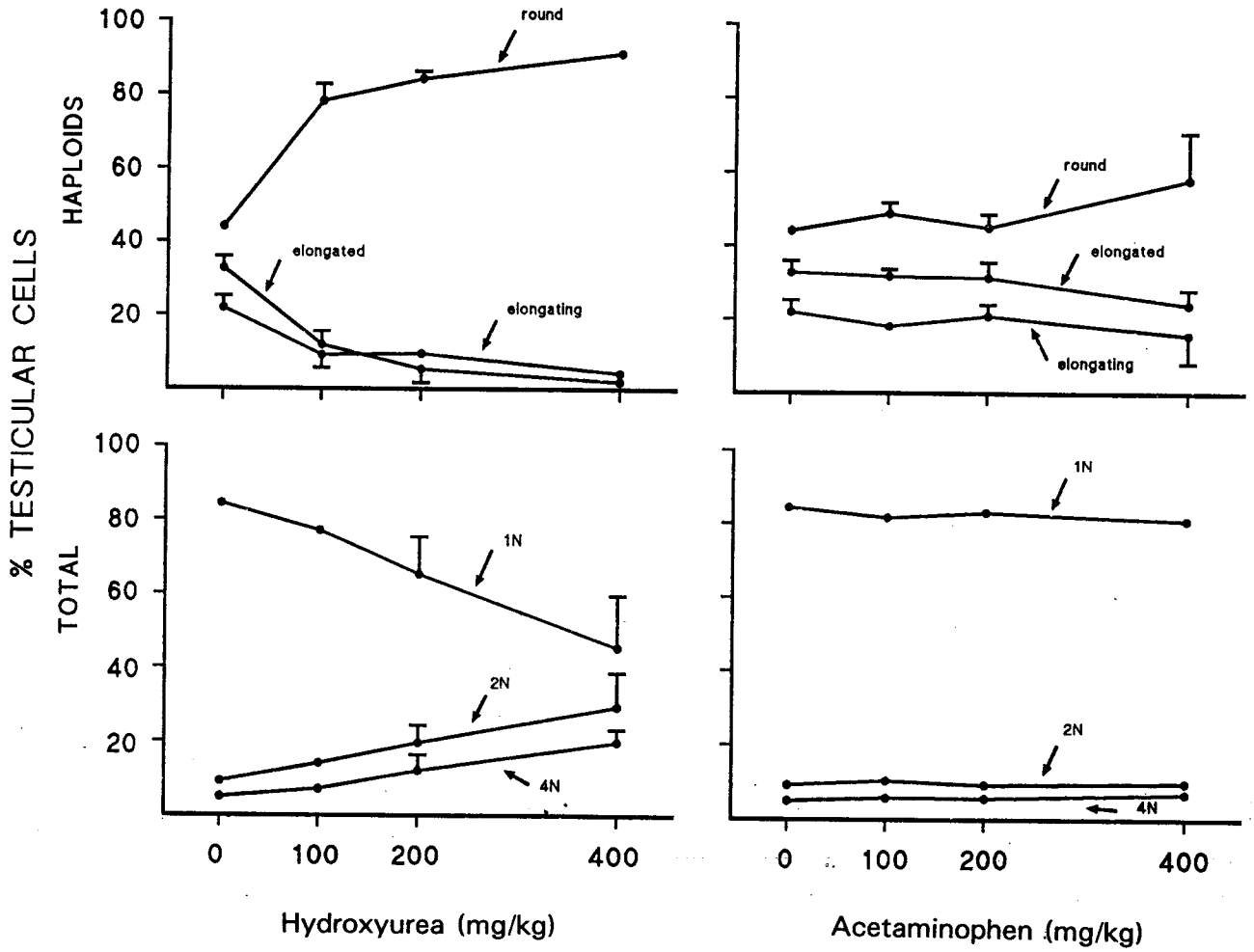

Fig. 6. Effects of various doses of acetaminophen or hydroxyurea on relative percentages of five testicular cell types in mice 26 d after last exposure. Mice received i.p. injections daily for 5 d. Mean values ± SD.

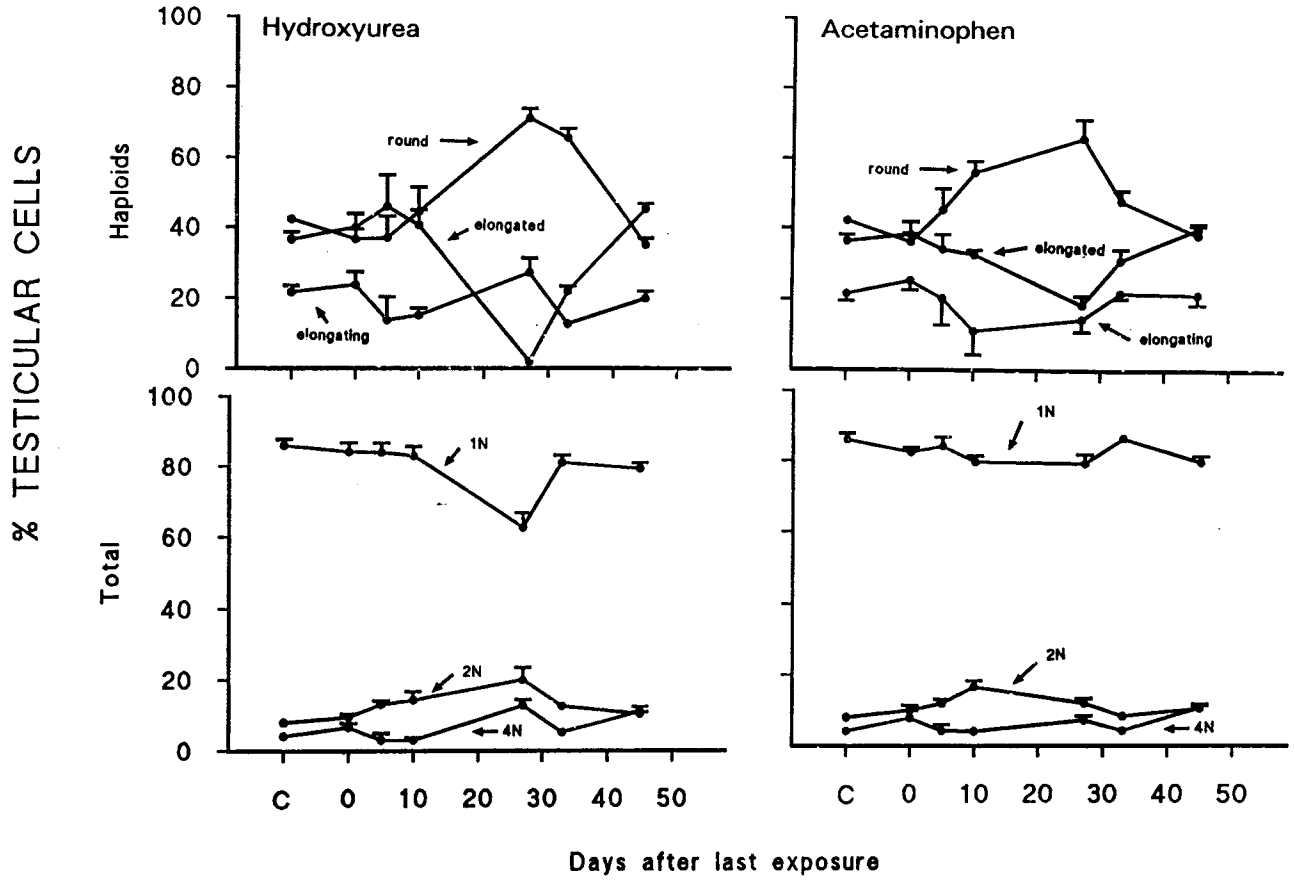

Fig. 7. Effects of acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg) on relative percentages of five categories of testicular germ cells in mice during various days after last treatment. Mice received i.p. injections daily for 5 d. Mean values ± SD.

(box 2) of the tetraploid cells, which were reduced from approximately 6% in the controls to less than 1% in the treated animals (Figure 5). Treatment also led to reductions in the proportions of late pachytene spermatocytes (box 1). The proportions of haploid and diploid cells were similar in all three groups.

Table 4. Alterations in the proportions of diploid and tetraploid populations from mouse testes during the 45-d period following treatment with acetaminophen (400 mg/kg) or hydroxyurea (200 mg/kg)

	Acetaminophen		Hydroxyurea	
Day	Diploid	Tetraploid	Diploid	Tetraploid
0	86 ± 12	113 ± 27	82 ± 7*	96 ± 17
5	84 ± 6	36 ± 14*	94 ± 5	26 ± 14*
10	109 ± 11	42 ± 19*	94 ± 15	64 ± 7*
27	148 ± 17*	182 ± 39*	248 ± 43*	322 ± 39*
33	103 ± 12	108 ± 20	155 ± 18*	132 ± 21
45	105 ± 8	113 ± 11	104 ± 5	113 ± 13

Values are expressed as percent of concomitant control values. (The number of cells in the diploid and tetraploid populations was approximately 450 and 250, respectively.)
*Significantly different ($P < 0.05$) from the control group by Student's t test.

Treatment-related alterations in the proportions of haploid, diploid, and tetraploid testicular cells were still evident on day 27. Hydroxyurea caused greater effects than acetaminophen, but both compounds caused statistically significant decreases in the haploid and increases in both the diploid and tetraploid populations compared to controls. An analysis of the haploid stages revealed that both treatments caused increases in the percentage of round spermatids, decreases in the elongated, and furthermore hydroxyurea also led to an increase in the proportion of elongating spermatids (Figure 7, Table 4).

In the 33-d samples the hydroxyurea-related alterations in the proportions of haploid and diploid populations that were observed on day 27 revealed changes toward the control values, but the increase in the diploid population of the hydroxyurea-treated mice remained significantly different (Table 4). By

45 d after treatment, the relative ratios of testicular cells had returned to normal values.

Flow cytometry of AO-stained sperm, which are first treated with acid to potentially induce DNA denaturation, has been used to study alterations in chromatin structure (32,35). Table 5 shows that five daily treatments with either hydroxyurea (200 mg/kg) or acetaminophen (400 mg/kg and 500 mg/kg) caused an increased frequency of cells with altered sperm chromatin structure 27 and 33 d after the last exposure.

DISCUSSION

It is well recognized that high doses of acetaminophen may cause hepatic necrosis and renal failure. Acetaminophen is activated by a direct two-electron oxidation of acetaminophen to *N*-acetyl-*p*-benzoquinoneimine (NAPQI) by cyt P-450 1A2, 2E1, and 3A4 (36–39) and/or alternately, by a one-electron oxidation to *N*-acetyl-*p*-benzosemiquinone imine by peroxidase, prostaglandin H synthetase, or cyt P-450 (40,41). The reactive metabolite causes depletion of reduced glutathione and arylated/oxidized macromolecules, thereby inducing cell death (13).

An evaluation of the effects of treatment on body weight revealed an initial weight loss in the acetaminophen-treated mice (Figure 1). This phenomenon has previously been reported (21). In the NTP study, mice that recieved 25,000 ppm (~462 mg/kg) *daily* in feed decreased from their initial weights during the first week of the study and then began to increase. After the initial period there were no significant differences in the growth rates between the three groups.

Acetaminophen-induced testicular toxicity has previously been observed in mice (19) and in rats (20,21). In the present study, both acetaminophen and hydroxyurea caused reductions in the absolute and relative testicular weights (Table 1). On day 33 after exposure, both 200 mg/kg hydroxyurea and 500 mg/kg acetaminophen caused statistically significant reductions in absolute and relative testicular weight. Five days after the last treatment the hydroxyurea-treated mice showed testicular atrophy of several seminiferous tubules, some of which were virtually devoid of germinal epithelium and contained mostly Sertoli cells (Figure 2). On the other hand, acetaminophen did not lead to such dramatic histologic changes. However, subtle but statistically significant alterations could been seen in testes on day 5, such as a decrease in the diameters of the seminiferous tubules. Drug metabolizing enzyme activity is found in the testis (42–44); however, compared to the liver, the relative level of P-450 vs. glutathione transferase and glutathione is low in the testis. Therefore, one would not expect the formation of acetaminophen adducts in appreciable quantities. The half-life of NAPQI has been found to be less than 15 sec in the presence of nucleophiles and reductants (45). Therefore, it is not likely that reactive metabolites formed in the liver are transported to the testicular cells. In accordance with this principle, we found that exposure to acetaminophen caused neither a depletion of glutathione (nonprotein sulphydryls) in the testis nor a marked increase in covalent binding (Tables 2 and 3). Thus, one should consider the possibility that testicular toxicity was induced by a mechanism different from the formation of a reactive metabolite.

Table 5. Effects of acetaminophen and hydroxyurea on percentage of vas deferens sperm with abnormal sperm chromatin structure

	Days after last exposure			
Treatment	5	10	27	33
Controls[a]	4.4 ± 0.7 (8)	4.4 ± 0.7 (8)	4.4 ± 0.7 (8)	4.4 ± 0.7 (8)
Hydroxyurea (200 mg/kg)	8.6 ± 1.7* (3)	6.9 ± 1.8 (4)	17.8 ± 3.9* (3)	26.9 ± 11.0* (3)
Acetaminophen (400 mg/kg)	4.5 ± 1.2 (3)	4.0 ± 0.2 (3)	8.8 ± 1.3* (4)	9.7 ± 2.2* (4)
Acetaminophen (500 mg/kg)	ND	ND	ND	11.0 ± 3.2* (3)

The effect of five daily treatments with acetaminophen or hydroxyurea on the chromatin structure of vas deferens sperm from mice using dual-parameter (green fluorescence (native DNA) vs. red fluorescence (denatured DNA)) flow cytometry of cells stained with acridine orange (mean % abnormal α_t ± SE).
*Significantly different ($P < 0.05$) from controls by Student's t test, ND = no data.
[a]The control values were pooled since all of the samples were analyzed on the same day.

Acetaminophen causes an inhibition of both DNA replication (16–18) and DNA repair synthesis (45) by a specific inhibition of ribonucleotide reductase. These effects provide an explanation for acetaminophen's ability to induce sister chromatid exchanges, micronuclei, and chromosomal aberrations in cells with low drug metabolism activity. Furthermore, preliminary data on HL-60 cells indicate that acetaminophen also may cause cell death (apoptosis) through an inhibition of DNA replication (unpublished data). Similar mechanisms could be involved in the toxicity observed in testicular cells (Table 1) (19,46). The results from the present study show that acetaminophen treatment, indeed, was able to interfere with thymidine incorporation in the testes of mice for several hours (Figure 6). Thus, most probably unmetabolized acetaminophen interferes with replicative DNA synthesis in these cell types. Spermatogonia and primary spermatocytes do not contain P-450 enzymes or peroxidases (42).

With regard to the composition of testicular cells, the greatest reductions in the tetraploid populations occurred on days 5 and 10 for both compounds, whereas the diploid populations remained similar to control values. Not all of the tetraploid cells were affected to the same extent. Cells gated into box 1 are likely to be late pachytene spermatocytes, while those in box 2 are mostly early pachytene spermatocytes and, to a certain degree, also type B spermatogonia (34). As can be seen in Figure 5, the greatest reductions can be seen in early pachytene spermatocytes (box 2), followed by late pachytene spermatocytes (box 1), with hydroxyurea producing the greatest effects. Accordingly, DNA synthesis was likely inhibited in type B and differentiating spermatogonia. In mice, passage from type B spermatogonia (diploid) to leptotene spermatocytes (tetraploid) takes 2.86 d (all times referred to here are from Oakberg [47]). This suggests that the interference in DNA synthesis that was observed (Figures 3 and 4) caused a marked reduction in the recruitment to leptotene spermatocytes by slowing down the cell cycle and possibly killing cells in the S phase. The relative ratios of the other populations remained nearly the same, suggesting that cellular differentiation proceeded on a near normal schedule following exposure, as indicated by the increased proportion of diploid cells on day 27. This action, thus, leads to a depletion of meiotic daughter cells and interferes with the flow of spermiogenesis. Acetaminophen did not appear to kill the spermatogonia stem cells which, following clearance of this compound, repopulate the germ cells with normal differentiation kinetics, as can be seen in Figure 7 (24). On the other hand, in two of four animals on day 10, hydroxyurea led to a loss of germinal epithelium in some of the tubules, which appeared to contain mostly Sertoli cells (Figure 2).

As would be expected, changes in the proportions of the various spermatid stages occurred later, and on day 27 the acetaminophen-treated mice revealed a great increase in the proportion of round spermatids and decrease in elongated spermatids (Figure 7). These changes are, in part, due to the very significant increase in tetraploid and diploid cells present as part of a new wave of spermatogenesis following exposure. Relative testis weights were still reduced by about 14% in the acetaminophen-treated mice compared to control values (Table 1). By day 45, the proportions of the testicular cell types had returned to near normal values in the acetaminophen-treated mice, but hydroxyurea delayed normalization of the composition of haploid cells for an additional several days (Figure 6).

Previous studies have shown that alkylating agents cause sperm chromatin structure abnormalities when measured as increased susceptibility towards DNA denaturation in situ (31,35). After exposure to methyl methanesulfonate, changes in chromatin structure can be observed within 3 d following a single exposure, indicating alkylation of free-SH groups on protamines in a late stage of spermatogenesis (48). On the other hand, ethylnitrosourea (31) targets early stages of spermatogenesis, most probably through reactions with DNA. Furthermore, inhibition of tubulin polymerization and microtubule formation may cause significant alteration of sperm chromatin structure (49). In the present study, indications of abnormal chromatin structure were found in vas deferens sperm, 27 and 33 d after the last acetaminophen exposure. Up to 10% of the sperm showed increased susceptibility towards DNA denaturation in situ (Table 5). These sperm cells were in the preleptotene stage at the beginning of exposure and approximately in the pachytene stage at the end of exposure, i.e., they were replicating DNA during the beginning of acetaminophen treatment.

Five and 10 d after acetaminophen exposure, vas deferens sperm showed no such increased susceptibility to DNA denaturation. These sperm were elongated spermatids to caput epidiymal at the end of exposure and elongating spermatids at the beginning of exposure (24). Thus, acetaminophen had no effect on late maturation of sperm cells.

It is important to note also that compounds that interfere with DNA synthesis can cause abnormal sperm chromatin structure [Table 5, (24)]. It has

been suggested from other studies on bulls (50) and humans (Evenson, in preparation) that these sperm are infertile. Ward and Coffey (51) have speculated that sperm must have a proper chromatin organization to function correctly for fertilization and early embryo development. This study indicates that acetaminophen interferes with testicular cell DNA synthesis, likely by the inhibition of ribonucleotide reductase, leading to a reduction in the normal deoxyribonucleotide pool size of spermatogonia and pachytene spermatocytes. Inhibited DNA synthesis has been reported to preclude the normal sequence of chromosome condensation in mammalian somatic cells (52). Accordingly, incomplete DNA replication or DNA repair synthesis may lead to an increased frequency of cells with apparent changes in sperm chromatin structure.

The link between inhibition of replicative DNA synthesis and ultimate changes in the sperm chromatin structure is not known. If the inhibition of DNA synthesis caused incomplete DNA replication, this could lead to an increased susceptibility towards DNA denaturation. During S-phase and in the early phases of sperm development histones are important in the packing of DNA. It is well documented that histone biosynthesis and DNA replication occur concomitantly during the S-phase of the cell cycle of many dividing cells (53). Thus, DNA inhibitors such as hydroxyurea cause a rapid reversible inhibition of histone synthesis (53), resulting in structural reorganization of chromatin (54). Furthermore, Smith et al. (55) reported that a hepatotoxic dose of acetaminophen in mice was accompanied by a dramatic decrease in mRNA for histone. Thus, it is possible that the altered chromatin structure observed in the vas deferens sperm could be related to errors in histone synthesis and chromatin structure in connection with the inhibition of replicative DNA synthesis that was observed in the testicular cells. During spermiogenesis, the histones are replaced by protamines, resulting in very tightly packed DNA (51). Immature sperm in the ejaculate usually have an abnormal exchange of histones for protamines, resulting in an increased stainability (increased green fluorescence) that is not seen in sperm from acetaminophen- or hydroxyurea-treated animals. Thus, the increased susceptibility towards chromatin denaturation seen in vas deferens sperm from drug-treated mice is probably not due to an increased proportion of immature sperm, but is rather an effect of chemical treatment on the packaging of chromatin.

In conclusion, five daily treatments with acetaminophen (400 mg/kg) caused an inhibition of DNA replication that was followed by a reduction in early pachytene spermatocytes and a reduced relative testicular weight. Ultimately, an increased frequency of sperm characterized by changes in chromatin structure, expressed as an increased susceptibility towards DNA denaturation in situ, as observed in vas deferens sperm 27 and 33 d after the last exposure.

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