Journal of Andrology, Vol. 25, No. 3, May/June 2004

Spermiogenic Germ Cell Phase–Specific DNA Damage Following Cyclophosphamide Exposure

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ABSTRACT: The production of genetically competent spermatozoa is essential for normal embryo development. The chemotherapeutic drug cyclophosphamide creates cross-links and DNA strand breaks in many cell types, including germ cells. This study assessed the phase specificity of the susceptibility of spermiogenic germ cells to genetic damage induced by cyclophosphamide. Adult male rats were given cyclophosphamide using one of four schedules: 1) high dose/acute—day 1, 100 mg/kg; 2) low dose/subchronic, 4 days—days 1–4, 6.0 mg/kg/d; 3) high dose/subchronic, 4 days—day 1, 100 mg/kg, and days 2–4, 50 mg/kg/d; and 4) low dose/chronic—daily, 6.0 mg/kg/d for 14–28 days. To capture cauda epididymal spermatozoa exposed to cyclophosphamide during late, mid-, and early spermiogenesis, animals were sacrificed on days 14, 21, and 28, respectively. Spermatozoa were analyzed for DNA strand breaks using the comet assay. No dramatic increases in damage were seen after high-dose/acute exposure to cyclophosphamide. Subchronic exposure showed a dose-related increase in DNA damage; maximal damage, as demonstrated by comet tail parameters, was seen after 21 days, reflecting an increased susceptibility of step 9–14 spermatids. Low-dose chronic exposure to cyclophosphamide induced DNA damage, which reached a plateau by day 21. The magnitude of damage at all time points after low-dose chronic exposure was much greater than that following low-dose exposure for 4 days, indicating an accumulation of damage over time. Thus, the DNA damage induced by cyclophosphamide is germ cell phase–specific. The most damaging effects of cyclophosphamide occurred during a key point of sperm chromatin remodeling (histone hyperacetylation and transition protein deposition). We speculate that strand breaks disrupt chromatin remodeling, hence affecting chromatin structure and embryo development.

Key words: Sperm, comet assay, chromatin remodeling, susceptibility, toxicology.

J Androl 2004;25:354–362

Society has growing concerns about the consequences of exposure to drugs and toxicants on the reproductive system, germ cells, fertilization, and development. Preconceptional exposure to drugs, radiation, or chemicals results in embryo loss, birth defects, and predisposition to cancer (Olshan and Mattison, 1994; Brinkworth, 2000; Robaire and Hales, 2003). Paternal exposure to toxicants may cause changes in sperm quality, thereby contributing to infertility and adverse progeny outcomes. Evidence is now accumulating about the importance to embryogenesis of genetically competent spermatozoa during both natural and assisted fertilization. Several authors have reported significant correlations between sperm DNA damage and effects on fertilization as well as embryo cleavage and pregnancy rates (Sun et al, 1997; Lopes et al, 1998b; Larson et al, 2000; Morris et al, 2002).

Among the commonly used anticancer drugs, alkylating agents have been associated most often with the development of infertility (Schilsky, 1980; Pont, 1997). Cyclophosphamide is a cytotoxic alkylating agent widely used in chemotherapeutic regimens. Its cytotoxic effects are the result of chemically reactive metabolites that create DNA adducts, DNA-DNA and DNA-protein cross-links, sister chromatid exchanges, chromosomal aberrations, and DNA strand breaks (Sotomayor and Cumming, 1975; Bishop et al, 1997). Exposure of male germ cells to cyclophosphamide leads to single-strand breaks, cross-links, and altered in vitro spermatozoal decondensation and template function (Qiu et al, 1995a,b). With little effect on the male reproductive system, chronic paternal treatment of rats with cyclophosphamide results in increases in embryo death and growth-retarded and malformed fetuses (Trasler et al, 1985, 1986, 1987; Jenkinson and Anderson, 1990). Although proliferating premeiotic germ cells are sensitive to alkylating agents, nondividing postmeiotic spermatids and spermatozoa are the most susceptible to DNA damage, leading to dominant lethality, heritable translocations, specific locus mutations, and malformations (Jackson, 1964; Ehling et al, 1968). Certain monofunctional alkylating agents, such as methyl methanesulfonate and ethylene oxide, produce more dominant lethal mutations and translocations in late spermatids and early spermatozoa than in any other germ cell stage (Ehling, 1980; Generoso et al, 1980). In contrast, other chemicals, such as ethyl nitrosourea and methyl ni-

Supported by the Canadian Institutes of Health Research MOP-10462.

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Received for publication November 6, 2003; accepted for publication December 8, 2003.

trosourea, show no germ cell stage–specific selection, with a wide range of effects on both pre- and postmeiotic germ cells (Russell et al, 1979; Sega et al, 1981). Cross-linking agents are also active throughout spermatogenesis; however, maximal genetic damage has been observed in early round spermatids (Russell, 1989, 1992). Dramatic increases in postimplantation loss are dose-dependent and maximal after a 3-week treatment of male rats with a low dose of cyclophosphamide (Trasler et al, 1985, 1986, 1987), which thereby exposes germ cells during spermiogenesis and sperm maturation.

Abnormal sperm chromatin packaging, identified as poorly protaminated spermatozoa or a high susceptibility of DNA to acid-induced denaturation using the sperm chromatin structure assay, has been correlated with the presence of DNA strand breaks (Gorczyca et al, 1993; Manicardi et al, 1995, 1998; Sailer et al, 1995). Mature spermatozoa contain highly packaged chromatin organized in a specific manner to allow access to required genetic information during embryogenesis. The proposed model for sperm DNA packaging consists of several levels of organization, including DNA loop domains attached to the nuclear matrix in a sequence-specific manner (Ward and Coffey, 1991; Ward, 1993) and protamine DNA binding (Balhorn, 1982). With such an intricate organization of the sperm nucleus, the possibility exists that damage to the DNA will, in turn, affect overall nuclear organization. Continuous treatment with cyclophosphamide for 4 weeks targets the male germ cell through all stages of spermiogenesis and sperm maturation. The adverse progeny outcomes associated with paternal exposure to this drug may be due to the accumulation of DNA damage in male germ cells as round spermatids develop into spermatozoa or due to spermiogenic germ cell phase–specific susceptibility to damage.

Genotoxic damage in the form of single- and double-stranded breaks can be measured using the comet assay. This assay is used extensively to assess DNA damage in somatic cells and has been applied to human and murine spermatozoa (Singh et al, 1989; Haines et al, 1998; Tice et al, 2000). Increased DNA damage in human spermatozoa measured by the comet assay is associated with infertility (Irvine et al, 2000) and exposure to chemotherapeutic agents (Chatterjee et al, 2000). In this study, we have developed the comet assay under alkaline conditions for rodent spermatozoa. We have used this assay to determine to what extent and at which point during spermiogenesis sperm acquire DNA damage after acute, subchronic, and chronic dosing regimens with cyclophosphamide.

Materials and Methods

Animal Treatments

Adult male Sprague-Dawley rats (400–450 g) were obtained from Charles River Canada (St Constant, Canada), maintained on a 14:10 light-dark cycle, and provided with food and water ad libitum. Rats were gavaged with saline or cyclophosphamide (CAS 6055-19-2; Sigma-Aldrich, Oakville, Canada) using one of four schedules: 1) high dose/acute—day 1, 100 mg/kg; 2) low dose/subchronic, 4 days—days 1–4, 6.0 mg/kg/d; 3) high dose/subchronic, 4 days—day 1, 100 mg/kg, and days 2–4, 50 mg/kg/d; and 4) low dose/chronic—daily, 6.0 mg/kg/d for 14–28 days. To capture spermatozoa first exposed to cyclophosphamide during late, mid-, and early spermiogenesis, animals were sacrificed by decapitation on days 14, 21, and 28 after the initiation of treatment (Clermont, 1972). Animal handling and care were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

Sperm Collection

To isolate spermatozoa from the cauda epididymides, the epididymides were first removed, trimmed free of fat, and washed in 2 mL of prewarmed (37°C) 10 mM Tris-HCl buffer containing 50 mM NaCl and 50 mM EGTA, pH 8.2–8.4. This medium maintains the genetic integrity of frozen spermatozoa (Kusakabe et al, 2001). The corpus–cauda epididymal junction was then clamped with a hemostat, and an incision was made in the distal cauda epididymidis. Spermatozoa released from the site of incision were collected in 2 mL of media at 37°C, incubated for 5 minutes to allow the spermatozoa to disperse, and then diluted 1:10 in fresh media. Aliquots of 100 μL were stored at −80°C until comet analysis was performed.

Detection of Damage in Individual Cells Using the Comet Assay

DNA damage in spermatozoa was evaluated using the comet assay as previously described (Singh et al, 1989; Haines et al, 1998) with the following modifications. Frozen sperm samples were thawed on ice and resuspended in Tris-HCl–buffered EGTA medium to a concentration of 1×10^5 cells/mL. Fifty microliters of the cell suspension was added to 500 μL of molten agarose (0.5% low-melting-point grade in Mg^{2+} and Ca^{2+}—free phosphate-buffered saline, pH 7.4, at 42°C). Sixty microliters was immediately pipetted and evenly spread onto slides (Trevigen Inc, Gaithersburg, Md), and the gel was allowed to solidify at 4°C in the dark for 10 minutes. All subsequent steps were performed under yellow light or in the dark to prevent any additional DNA damage.

Slides were immersed in prechilled (4°C) lysis buffer (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris-HCl; final pH, 10) containing 10% dimethylsulfoxide, 1% Triton X-100, and 40 mM dithiothreitol for 1 hour on ice. Following initial lysis, slides were washed in distilled water for 5 minutes. Proteinase K, at a concentration of 0.1 mg/mL, was added to fresh prewarmed lysis buffer, and additional lysis was performed for 3 hours at 37°C. Slides were washed in distilled water, placed flat at 4°C for 10 minutes to reset the agarose, and then immersed in freshly prepared alkaline solution (1 mM EDTA and 0.05 M NaOH, pH 12.1) for 45 minutes in the dark. Slides were washed twice in 1× Tris-Borate-EDTA buffer (TBE, pH 7.4) for 5 minutes and then placed equidistant from the electrodes on a gel tray submerged in TBE in a horizontal electrophoresis apparatus (Mini-Sub Cell GT; Bio-Rad Laboratories, Inc, Mississauga, Canada).

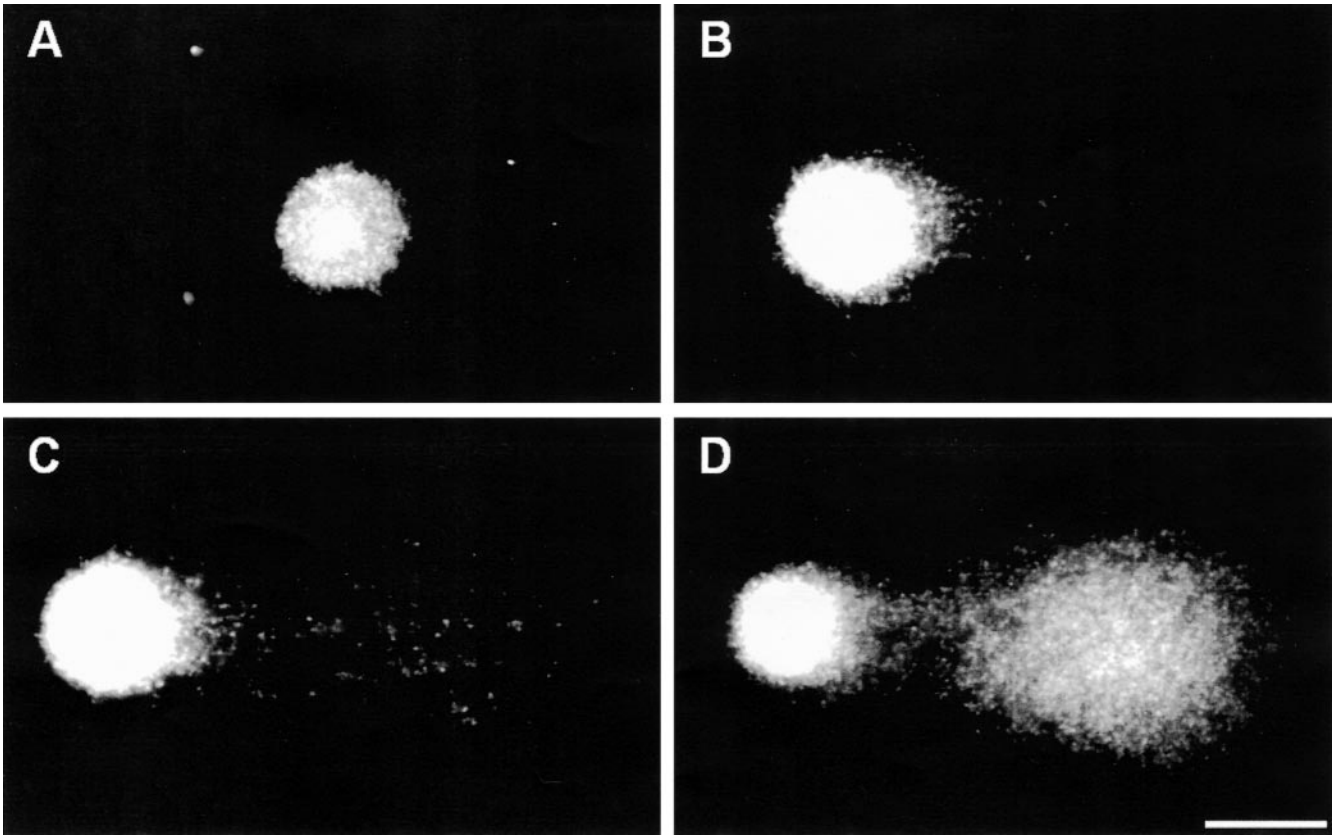

Figure 1. Images of spermatozoal nuclei following the comet assay. **(A)** Control sperm; **(B–D)** cyclophosphamide-exposed sperm. All images are at 200× magnification. Scale bar = 40 μm.

The level of TBE was 2–4 mm above the slides. Electrophoresis was carried out at 14 V (0.7 V/cm) for 10 minutes. Slides were drained and fixed in ice-cold 70% ethanol for 5 minutes and left to air dry prior to storage with desiccant at room temperature. Similar to the standard alkaline comet assay defined by Singh et al (1988), treatment with alkali to unwind and denature the DNA followed by electrophoresis in neutral buffer permits the detection of single-strand breaks. These conditions, however, tend to give lower background levels of DNA damage, result in better dose responses, and avoid saturating the assay so that estimation of damage is over a wider range (Koppen and Angelis, 1998; Angelis et al, 1999).

DNA was stained with 50 μL of SYBR Green solution (Trevigen) diluted 1:10 000 in Tris-EDTA buffer, pH 7.5, and immediately analyzed at 200× magnification using a DAGE-MTI CCD300-RC camera (DAGE-MTI Inc, Michigan City, Ind) attached to an Olympus BX51 epifluorescence microscope. Fifty cells were randomly analyzed per slide for a total of 100 cells per animal, and fluorescent images were scored for comet parameters. Tail length, percent tail DNA, and tail moment (tail length × fraction of tail DNA) were measured using the KOMET 5.0 image analysis system (Kinetic Imaging Ltd, Liverpool, United Kingdom).

Statistical Analysis

Significant differences due to drug treatment or time of drug exposure were determined using a 2-way analysis of variance followed by the Bonferroni post hoc test ($P < .05$). The percent abnormal sperm among populations of spermatozoa from saline- and cyclophosphamide-treated animals was compared by chi-square analysis. Statistical analyses were performed by the SigmaStat 2.03 software package (SPSS Inc, Chicago, Ill).

Results

In the alkaline comet assay, an increase in DNA strand breaks leads to greater DNA migration out of the nucleus and into the tail of the comet. The majority of control spermatozoa had little if any comet tail (Figure 1A). Differences in tail length and tail intensity were observed among cyclophosphamide-exposed sperm populations (Figure 1B through D). The extent of damage was dependent on dose as well as on time and length of exposure to cyclophosphamide.

Acute 1-Day Exposure

Exposure of male germ cells to a single high dose of cyclophosphamide resulted in time-specific increases in DNA damage (Figure 2). Small yet significant differences were observed only in the percentage of tail DNA values 14 days after drug administration (1.4-fold increase) and

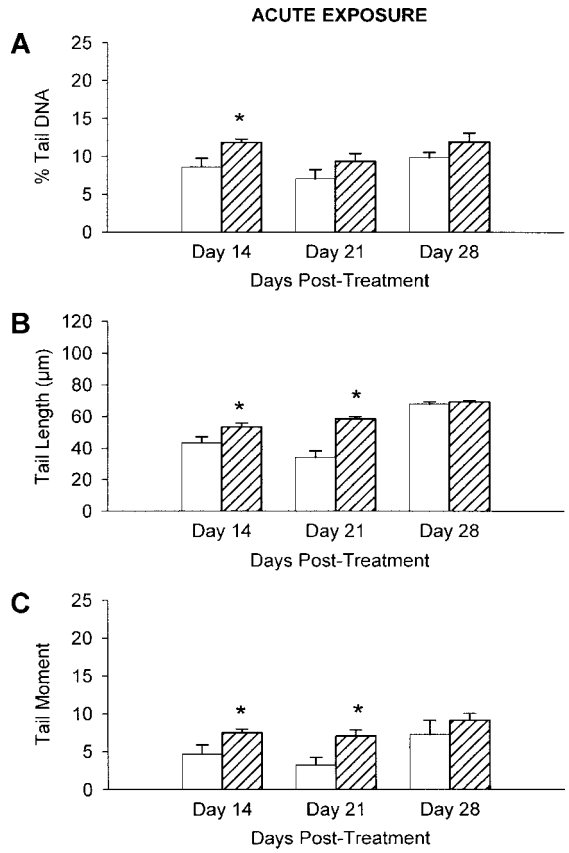

Figure 2. DNA damage assessed by the comet assay in epididymal spermatozoa on days 14, 21, and 28 after acute 1-day cyclophosphamide exposure. Effects measured as **(A)** % tail DNA, **(B)** tail length, and **(C)** tail moment are shown for saline (open bars) and cyclophosphamide-exposed (crosshatched bars) sperm populations. The data shown represent means ± SEM (n = 3). * Significantly different from time-matched controls ($P < .05$).

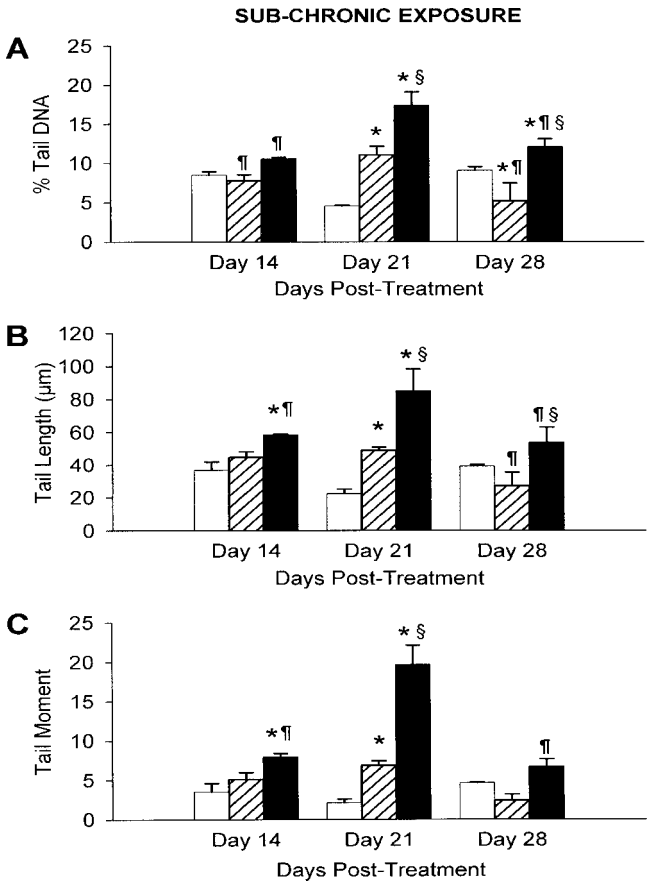

Figure 3. DNA damage assessed by the comet assay in epididymal spermatozoa on days 14, 21, and 28 after subchronic 4-day cyclophosphamide exposure. Effects measured as **(A)** % tail DNA, **(B)** tail length, and **(C)** tail moment are shown for saline (open bars), low-dose cyclophosphamide (crosshatched bars), and high-dose cyclophosphamide (black bars) sperm populations. Data shown represent means ± SEM (n = 3). * Significantly different from time-matched controls; § significantly different from time-matched low-dose cyclophosphamide group; ¶ significantly different from dose-matched day 21 cyclophosphamide group ($P < .05$).

in tail length values after 14 days (1.2-fold increase) and 21 days (1.7-fold increase). On the integration of these 2 comet parameters to obtain the tail moment, significant differences between drug-exposed and time-matched controls were observed after 14 days (1.6-fold) and 21 days (2.2-fold). Acute exposure to cyclophosphamide did not alter sperm DNA integrity after 28 days; this may indicate either a decrease in susceptibility to damage or the presence of repair in early spermiogenic germ cells.

Subchronic 4-Day Exposure

Short-term repeat exposure to cyclophosphamide resulted in dose- and time-dependent increases in DNA damage (Figure 3). By day 14, no significant differences were seen between sperm exposed to low doses of cyclophosphamide and control sperm for any comet parameters. Administration of a higher dose, however, resulted in significant differences in tail length (1.6-fold) and tail moment (2.3-fold). Drug-exposed spermatozoa collected on day 21 showed the highest levels of DNA strand breaks detected at any time point. Measurements of comet tail moment after exposure to low or high cyclophosphamide doses were 3.3- and 9.4-fold higher than for the control, respectively (Figure 3C). Similar changes were observed for the percentage of tail DNA and tail length; however, the magnitudes were not as great (low dose: 2.4- and 2.2-fold increases, respectively; high dose: 3.8-fold increase for both parameters). Spermatozoa collected on day 28 had relatively lower levels of DNA strand breaks than spermatozoa collected on day 21.

Chronic Exposure

Significant increases in the percentage of tail DNA, tail length, and tail moment were detected at all time points after chronic exposure to low doses of cyclophosphamide (Figure 4). There were significant yet very small differences in the percent tail DNA for cyclophosphamide-exposed sperm populations at the 3 time points. In contrast,

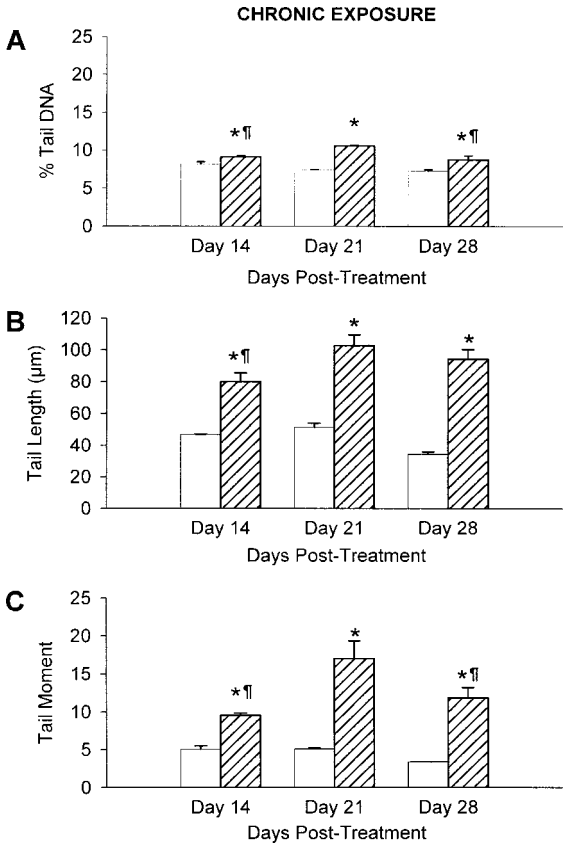

Figure 4. DNA damage assessed by the comet assay in epididymal spermatozoa on days 14, 21, and 28 after chronic cyclophosphamide exposure. Effects measured as **(A)** % tail DNA, **(B)** tail length, and **(C)** tail moment are shown for saline (open bars) and cyclophosphamide-exposed (crosshatched bars) sperm populations. Data shown represent means ± SEM (n = 4). * Significantly different from time-matched controls; ¶ significantly different from dose-matched day 21 cyclophosphamide group ($P < .05$).

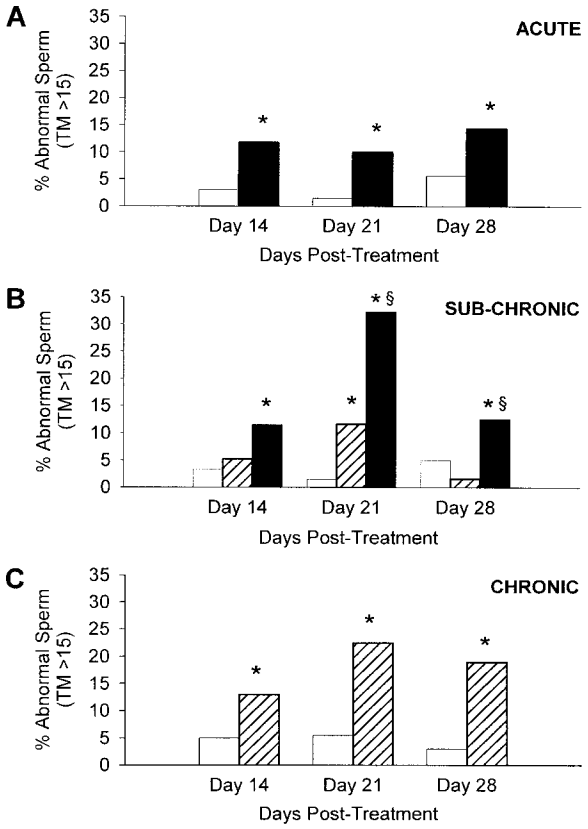

Figure 5. Percentage of epididymal spermatozoa with tail moments greater than 15. Cells were collected on days 14, 21, and 28 after **(A)** acute 1-day, **(B)** subchronic 4-day, and **(C)** chronic cyclophosphamide exposure and then analyzed for DNA damage measured as comet tail moment. The percentage of abnormal sperm presenting increased DNA damage is shown for saline (open bars), low-dose cyclophosphamide (crosshatched bars), and high-dose cyclophosphamide (black bars). TM indicates tail moment. * Significantly different from time-matched controls; § significantly different from time-matched low-dose cyclophosphamide group ($P < .05$).

the mean tail length showed a significant increase in damage from day 14 (80.1 μm) to day 21 (102.8 μm) and then a slight decrease by day 28 (94.2 μm). Tail moments showed a 1.9-fold increase in DNA damage after 14 days; damage was maximal by 21 days of chronic cyclophosphamide exposure (3.4-fold increase). In comparison to the mean tail moments observed after low-dose subchronic exposure (Figure 3C), the mean tail moment values for each time point were much higher after chronic drug exposure, indicating an accumulation of cyclophosphamide-induced damage during the entire course of drug administration. This increase in damage appeared to reach a plateau by day 21, as there was no difference between the fold increases in damage for days 21 and 28 (3.4- and 3.5-fold increases, respectively).

Distribution of Sperm From Low-Damage to High-Damage Groups

Frequency histograms of tail moment represent the distribution of sperm damage. Tail moments for drug-exposed spermatozoa had a heterogeneous distribution pattern, ranging from 0 to 100, rather than discrete populations of low and high DNA damage. Differences in the percentage of sperm with increasing amounts of damage were, however, still noticeable. An overwhelming majority (≥94%) of tail moments for control animals ranged from 0 to 15; therefore, in comparison, the percentage of abnormal sperm with tail moments greater than 15 in drug-treated populations was calculated (Figure 5).

Significant increases in the percentage of abnormal sperm were seen at all time points among the cyclophosphamide-treated group in the high-dose/acute study (Figure 5A). By day 14, 12% of sperm were damaged, while by day 21, 10% of sperm had tail moments above 15. Note that although no significant difference was found between tail moment means of drug-exposed and control sperm at day 28 (Figure 2C), there was still a higher number (14%) of sperm from cyclophosphamide-treated animals with abnormal DNA. For the subchronic cyclophos-

phamide study, the highest percentage of abnormal sperm was found within the population of cells collected after 21 days for both low and high cyclophosphamide doses (12% and 32%, respectively; Figure 5B). In the day 28 high-dose cyclophosphamide-exposed sperm, significant differences were found, even though the tail moment mean was not significantly different from controls. At days 14 and 28, only sperm collected after high-dose cyclophosphamide administration had a significantly higher than control percentage of sperm with increased DNA damage.

Chronic low-dose administration of cyclophosphamide resulted in a significant increase in the percentage of abnormal sperm compared to controls (Figure 5C). Only 13% of sperm presented with abnormal DNA (2.6-fold increase) by day 14. Cyclophosphamide-induced damage accumulated in 22% of the germ cells that were first exposed during mid-spermiogenesis and collected 21 days later (4.1-fold increase). The percentage of abnormal sperm did not further increase by day 28.

Discussion

Using alkaline elution, previous studies (Qiu et al, 1995b) have shown that 6 weeks of chronic cyclophosphamide treatment significantly increases both DNA single-strand breaks and cross-links in spermatozoa. Chronic exposure to cyclophosphamide, targeting spermatids and epididymal spermatozoa, resulted in increases in peri-implantation embryo loss and fetal growth retardation and malformations (Trasler et al, 1985, 1986, 1987). The goal of this study was to elucidate the extent of DNA damage in spermatozoa induced by cyclophosphamide at specific spermiogenic time points. The comet assay is a sensitive measure of DNA damage in individual sperm nuclei, thus making it possible to determine whether or not all cells within a population demonstrate the same degree of damage. Only a few studies have used the comet assay to assess genotoxic damage to male germ cells after in vivo exposure to a testicular genotoxicant (Anderson et al, 1996; Haines et al, 2001, 2002).

The germ cell phase most sensitive to the induction of mutations by cyclophosphamide was the early spermatid phase (Sotomayor and Cumming, 1975). As measured by the comet assay, the mid-spermiogenic germ cells were most susceptible to DNA damage after cyclophosphamide exposure. These data, and those of Haines et al (2002), on germ cell phase–specific susceptibility to radiation damage suggest that the comet assay is not predictive of subtle mutational changes. However, high amounts of DNA damage as detected by the comet assay have been associated with the failure of embryo development after intracytoplasmic sperm injection (Morris et al, 2002) and with infertility in humans (Irvine et al, 2000). The DNA damage measured by the comet assay may also provide an accurate reflection of adverse reproductive outcome after chronic low-dose administration of cyclophosphamide to male rats (Trasler et al, 1985, 1986, 1987).

Cyclophosphamide exposure resulted in dose-dependent and time-specific increases in the levels of DNA damage; as dose and exposure time increased, so did the fraction of cells with DNA damage. Acute exposure to a high dose of cyclophosphamide did not create any major elevations in damage, and the damage that was noted was readily reversible. Step 9 and 15 spermatids collected as spermatozoa on days 21 and 14, respectively, incurred only low levels of damage. Acute cyclophosphamide exposure affected gene expression most dramatically in round spermatids; the expression of DNA repair genes was induced (Aguilar-Mahecha et al, 2001). These data suggest that round spermatids attempt to compensate for DNA damage induced by the drug. Neither acute nor subchronic cyclophosphamide exposure resulted in a significant increase in damage in germ cells exposed to the drug as round spermatids (acute: step 1 spermatids, subchronic: steps 1–5 spermatids). Postmeiotic spermatids do not undergo apoptosis (Cai et al, 1997; Sinha Hikim and Swerdloff, 1999; Brinkworth and Nieschlag, 2000); however, early to mid-spermatids do have the ability to repair DNA lesions (Sega, 1976; Sotomayor et al, 1978) that may give rise to DNA strand breaks. The ultimate effect of cyclophosphamide on germ cells will depend on both the extent of damage and their ability to undergo DNA repair. While there was no significant increase in overall damage at this stage, there was still a discrete population of abnormal sperm presenting increased levels of damage, and these cells may be capable of affecting progeny.

Repeat exposure to cyclophosphamide was required for higher levels of damage to occur. Subchronic treatment of male rats produced maximal damage in elongating spermatids exposed to the drug from stages 9 to 14. Chronic administration of cyclophosphamide resulted in the greatest accumulation of damage over time; the damage reached a plateau after 21 days of drug treatment, at a time point when germ cells exposed to cyclophosphamide are developing from elongating spermatids (steps 9–19) to mature spermatozoa. Cyclophosphamide may exert its maximal effect on elongating spermatids and spermatozoa, since these cells have lost the ability to repair DNA and undergo apoptosis.

Differences in susceptibility to genetic damage between mid- (steps 9–14) and late (steps 15–19) spermatids may be due to chromatin structural changes during spermiogenesis. The structural organization of DNA in the sperm may determine the participation of the paternal genome in embryo development. During spermiogenesis, mammalian sperm DNA is reorganized into loop domains at-

tached at specific sites to the nuclear matrix (Ward and Coffey, 1991; Ward, 1993). The unique chromatin architecture that results may be required to facilitate scheduled transcription after fertilization. DNase-I-hypersensitivity regions have been localized to transcriptionally active or potentially active genes near nuclear matrix attachment sites (Gross and Garrard, 1988). The pattern of DNase-I-hypersensitivity regions changes during spermiogenesis; step 12–13 elongating spermatids are the most sensitive to enzymatic action (McPherson and Longo, 1992). This stage of spermiogenesis may represent a point at which the male genome is more accessible to insult. In comparison, alkylating agents preferentially damage DNA regions that are in close proximity to matrix-bound replication and transcription sites (Muenchen and Pienta, 1999). The template function of spermatozoal DNA was markedly affected after 6 weeks of treatment with cyclophosphamide (Qiu et al, 1995b). During spermiogenesis, certain gene loci may be more susceptible to cyclophosphamide alkylation; we hypothesize that these genes play an important role in regulating early embryo development.

Final packaging of the sperm DNA occurs as histone acetylation and ubiquitination increase in mid-spermiogenic spermatids to allow transition proteins to bind to the DNA. These transition proteins then facilitate the preferential binding of protamines in late spermatids to condense the chromatin (Poccia, 1986; Wouters-Tyrou et al, 1998). Endogenous nicks in DNA, present normally in step 12–13 rat spermatids, are ligated before the completion of protamination (McPherson and Longo 1992, 1993a,b; Sakkas et al, 1995). These nicks may be present to facilitate protamination by providing relief of tortional stress (McPherson and Longo, 1992). Improper ligation of these breaks may result in altered chromatin structure and residual DNA strand breaks in spermatozoa (Sailer et al, 1995; Caron et al, 2001; Kierszenbaum, 2001). The presence of DNA damage in mature spermatozoa has been correlated with poor chromatin packaging (Gorczyca et al, 1993; Sailer, 1995; Manicardi et al, 1998), possibly due to underprotamination (Manicardi et al, 1995; Sakkas et al, 1996). Cyclophosphamide may affect the relationship between protamination and endogenous nick creation and ligation. Thus, cyclophosphamide maximally damages DNA at its most vulnerable state, during mid-spermiogenesis, when nucleoproteins are involved in the histone-protamine exchange.

Decreased iodoacetamide binding, indicating decreased reducible sulfhydryl content of sperm nuclei, was observed in cyclophosphamide-exposed germ cells, suggesting that protamine is affected in these cells (Qiu et al, 1995a). Effects on reducible sulfhydryl content may be due to either incomplete protamine deposition or an increase in alkylation of protamine sulfhydryl groups. Protamines in the testis may be especially susceptible to alkylation; if so, the end result would be blockage of normal disulfide bond formation, thus preventing proper chromatin condensation. This could lead to stress in the chromatin structure and result in DNA strand breaks (Sega and Owens, 1983). Fertilization with damaged or poor-quality sperm may lead to aberrant pronuclear development (Lopes et al, 1998a). Indeed, male pronulear formation was early in rat oocytes sired by cyclophosphamide-treated males (Harrouk et al, 2000b). Sega and Owens (1983) have paralleled patterns of alkylation of protamine in late spermatids and early spermatozoa to the occurrence of dominant lethal mutations.

In summary, cyclophosphamide-induced DNA damage accumulates over time, with the most damaging effects occurring during mid-spermiogenesis. The step 9–14 spermatids appear to be most susceptible to DNA damage; this represents a key time point in sperm chromatin remodeling (histone acetylation and transition protein deposition). Fertilization can be achieved using damaged spermatozoa; however, proper embryo development depends on spermatozoal genetic integrity (Ahmadi and Ng, 1999). In previous studies, using the comet assay, we have shown that cyclophosphamide-exposed spermatozoa imparted significantly greater DNA damage to fertilized eggs at the single-cell stage than did control spermatozoa (Harrouk et al, 2000a). Given the results of the present study, we speculate that cyclophosphamide disrupts chromatin remodeling, hence affecting sperm chromatin structure and embryo development.

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