DNA Damage Recognition in the Rat Zygote Following Chronic Paternal Cyclophosphamide Exposure

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The detrimental effects of preconceptional paternal exposure to the alkylating anticancer agent, cyclophosphamide, include aberrant epigenetic programming, dysregulated zygotic gene activation, and abnormalities in the offspring that are transmitted to the next generation. The adverse developmental consequences of genomic instabilities transmitted via the spermatozoon emphasize the need to elucidate the mechanisms by which the early embryo recognizes DNA damage in the paternal genome. Little information exists on DNA damage detection in the zygote. We assessed the impact of paternal cyclophosphamide exposure on phosphorylated H2AX (γH2AX) and poly(ADP-ribose) polymerase-1 (PARP-1), biomarkers of DNA damage, to determine the capacity in the rat zygote to recognize genomic damage and initiate a response to DNA lesions. An amplified biphasic γH2AX response was triggered in the paternal pronucleus in zygotes sired by drug-treated males; the maternal genome was not affected. PARP-1 immunoreactivity was substantially elevated in both parental genomes, coincident with the second phase of γH2AX induction in embryos sired by cyclophosphamide-exposed spermatozoa. Thus, paternal exposure to a DNA damaging agent rapidly activates signals implemental for DNA damage recognition in the zygote. Inefficient repair of DNA lesions may lead to persistent alterations of the histone code and chromatin integrity, resulting in aberrant embryogenesis. We propose that the response of the early embryo to disturbances in spermatozoal genomic integrity plays a vital role in determining its outcome.

Key Words: histone modifications; developmental toxicity; embryo; spermatozoa; γH2AX; poly(ADP-ribose)lation.

An intact paternal genome is crucial for successful embryogenesis; subtle alterations in sperm nuclear organization disrupt genomic and epigenetic regulation during development (Barton et al., 2005; Harrouk et al., 2000b; Ward et al., 2000). Preconceptional exposure of male rodents to chemotoxicants or irradiation leads to the transmission of chromosomal aberrations (Marchetti et al., 2004), germ-line instabilities (Barber et al., 2002, 2006), and a variety of abnormalities (Auroux et al., 1990; Hales et al., 1992) in the offspring that may persist for multiple generations.

Cyclophosphamide, a cytotoxic alkylating agent used to treat various cancers and autoimmune diseases, targets rapidly dividing cells, inducing DNA cross-links and DNA single-strand breaks (Colvin, 1999). Spermatogenesis is a complex, sequentially ordered process rendering male germ cells particularly susceptible to genotoxic agents. Chronic preconceptional exposure of males to cyclophosphamide generates genetic instabilities in spermatozoa (Cordrington et al., 2004) that are introduced into the oocyte at fertilization (Harrouk et al., 2000a).

Sophisticated mechanisms to recognize and repair DNA damage have been described extensively in eukaryotic cells (Nordstrand et al., 2007). In contrast, male germ cells lack an effective repair system to eliminate lesions incurred during spermiogenesis and spermatozoal maturation (Cordrington et al., 2004; Spermon et al., 2006); disruptions in the integrity of sperm DNA must be resolved early postfertilization to ensure faithful transmission of genetic information throughout embryogenesis. Following fertilization with mutagen-exposed spermatozoa, the egg has basic DNA repair capacity (Generoso et al., 1979). Additionally, mRNA analyses provide evidence for the expression of numerous repair genes during early development (Harrouk et al., 2000a; Zheng et al., 2005); however, there is very limited knowledge of the mechanisms implemented by the mammalian zygote in response to paternally transmitted DNA damage.

Histone phosphorylation and poly(ADP-ribosyl)ation are well-characterized posttranslational modifications implemental in the maintenance of genomic stability (Celeste et al., 2002; Masutani et al., 2000). Histone H2AX is rapidly phosphorylated at sites of DNA double-strand breaks; phosphorylated H2AX (γH2AX) subsequently recruits numerous essential repair proteins to the vicinity of the DNA lesions (Paull et al., 2000). In addition to a prominent role in the genotoxic stress response in mid-spermatogenic germ cells (Aguilar-Mahecha et al., 2005), γH2AX foci are observed coincident with major chromatin remodeling events in the testis (Hamer et al., 2003), coalesce in the condensing X-Y body of spermatocytes...
confering transcriptional inactivation (Fernandez-Capetillo et al., 2003), and to have female-specific roles in chromosomal dynamics of the oocyte (Roig et al., 2004). Collectively, these sites emphasize the importance of H2AX phosphorylation as a histone posttranslational modification implemental in the control of chromatin modifications and cellular functions. Interestingly, γH2AX signaling has been observed immediately after gamete fusion in the mouse zygote (Derijck et al., 2006).

Poly(ADP-ribosyl)ation of nuclear proteins plays a fundamental role in the detection of DNA strand breaks, the recruitment of repair factors to the lesion site, and the regulation of chromatin structure (Schreiber et al., 2006). Poly(ADP-ribose) polymerase-1 (PARP-1) catalyzes poly (ADP-ribosylation), binds with high affinity to DNA strand breaks, and is an integral mediator of DNA base excision repair and single-strand break repair (de Murcia et al., 2003). Although poly(ADP-ribosylation) has been linked to chromatin remodeling events in the preimplantation embryo (Imamura et al., 2004), the kinetics of PARP-1 activation in response to male-mediated genotoxic stress have not been reported. We propose that the DNA damage incurred in the paternal genome due to preconceptional cyclophosphamide exposure activates early damage responses that are essential for the detection of DNA strand breaks in the zygote.

MATERIALS AND METHODS

Drug regime, in vivo rat embryo generation, and collection. Sprague-Dawley rats were purchased from Charles River Canada (St Constant, Quebec, Canada) and housed at the Animal Resources Centre, McIntyre Medical Building, McGill University. Animal housing conditions, treatments, and embryo protocols were done following the procedures previously described (Barton et al., 2005). Briefly, adult male rats (body weight, 350–400 g) were gavaged with saline or 6 mg/kg/day of cyclophosphamide (CAS 6055-19-2; Sigma Chemical Co., St Louis, MO) six times per week for 4–5 weeks (Harrouk et al., 2000a,b). The selected treatment regime ensures that the embryos are sired by spermatooza that were initially exposed during the highly sensitive phase of spermiogenesis, encompassing sperm chromatin organization and packaging (Codrington et al., 2004). Early in the fifth week of treatment, each male was mated overnight with two virgin females (body weight, 225–250 g) in proestrus; pregnancies were confirmed the following morning, designated gestation day 0. Sperm-positive females were euthanized at 1300 h; oviducts were washed in 1% hyaluronidase (Sigma Chemical Co.) for cumulus cell dissociation. Zygotes in proestrus; pregnancies were confirmed the following morning, designated gestation day 0. Sperm-positive females were euthanized at 1300 h; oviducts were washed in 1% hyaluronidase (Sigma Chemical Co.) for cumulus cell dissociation. Zygotes were incubated in 1× phosphate-buffered saline (PBS), pH 7.4 (Mg2+ and Ca2+ free), containing 1 mg/ml polyvinylpyrrolidone; zona pellucidae were removed subsequently by briefly rinsing embryos in a drop of acid Tyrode’s solution. Embryos were prepared for immunofluorescence staining as described below. Six experimental replicates were done for the assessment of PARP-1, respectively. DNA was stained with 10 μg/ml 4’, 6-diamidino-2-phenylindole (DAPI) for 30 min, and embryos were washed and mounted in 3 μl of Vectashield antibleaching mounting medium (Vector Laboratories) on premarked slides.

Antibodies. Primary antibodies were mouse monoclonal IgG1 anti-γH2AX (phospho-Serine-139), clone JBW301 (1:500 dilution; catalog number 05-636, Upstate Biotechnology, Charlotte, NC), and mouse monoclonal IgG1 anti-PARP-1, clone C-2-10 (1:200 dilution; catalog number AM30, Calbiochem, San Diego, CA). Secondary antibodies used for immunofluorescence detection were sheep anti-mouse fluorescent-conjugated IgG (1:200 dilution; catalog number N1031, Amersham Pharmacia Biosciences, Baie d’Urfe, Quebec, Canada) and horse anti-mouse Texas Red IgG (1:200 dilution; catalog number TI-2000, Vector Laboratories). Nuclear staining specificity was confirmed only by secondary antibody incubations. The second polyclonal antibody to be used for a internal staining control for γH2AX as it is highly stained during cytotrophic development.

Confocal microscopy. Optical z-sections of individual embryos were recorded using a Zeiss LSM 510 Axiovert 100M confocal microscope equipped with a Plan-Apochromat X63/1.4 oil DIC objective. Prior to extensive imaging, optimal conditions for laser scanning confocal microscopy were established experimentally; z-stacks were captured using identical parameter settings for all embryos. Fluorescein, Texas Red, and DAPI fluorochromes were excited by 488, 543, and 730 nm (2-photon) lasers, respectively; multitracking was used to allow sequential imaging of individual fluorescence emissions avoiding cross talk between channels. Pinhole diameters were set at 0.84 and 1.00 Airy units, resulting in individual optical slices of 0.6 or 0.8 μm for γH2AX and PARP-1, respectively. Images were scanned at a speed of 6 (pixel time of 3.20 μs) with an average of two scans per optical section, in 1024 × 1024 pixels for an optimal resolution of 0.14 × 0.14 μm pixel size. Each data stack was acquired at a zoom factor equal to one using detector gain settings of 1150 and 1050 for γH2AX and PARP-1, respectively. Photobleaching of fluorescent signals was avoided by scanning a single embryo within a specified boundary one time only. Digital images were collected and transferred into appropriate file formats for respective analytical imaging software. In accordance with our previous classification criteria (Barton et al., 2005), zygotes were categorized qualitatively into five pronuclear stages (PN) based on interpronuclear positioning of parental genomes and morphological assessment of the paternal pronucleus.

Quantification of γH2AX. Image stacks generated by laser scanning confocal microscopy were imported into IMARIS (Bitplane AG, Zurich, Switzerland), an automated imaging software for detailed processing of multidimensional images. γH2AX staining in male and female pronuclei was observed to be qualitatively different; the 3D reconstruction of each data set was cropped and parental pronuclei processed separately. γH2AX foci were quantified independently using IMARIS Spots and Isosurface measurement modules which provide a detailed comparative analysis of each distinct 3D focus. Prior to automated focus detection, image stacks were smoothed using a Gaussian filter; optimal threshold limits were set to eliminate insignificant background objects. Using the spots module, foci within the selected detection range (minimum diameter, 0.200 μm; maximum diameter, 5.00 μm; threshold, 25) were modeled as spherical structures, each belonging to a spatial position along the x-, y-, and z-axis; the calculated sum number of foci per pronucleus

otherwise indicated; all washes were done in 0.05% Tween 20 in PBS for 5 min. Zygotes were fixed in 4% paraformaldehyde for 15 min, washed, permeabilized in PBS containing 0.2% Triton X-100 for 30 min, rewarmed thoroughly, and blocked overnight at 4°C. Embryos to be used for γH2AX detection were blocked in a solution of 10% goat serum (Vector Laboratories, Burlington, Ontario, Canada), 2% bovine serum albumin, and 0.05% Tween 20 in PBS; goat serum was omitted from the blocking solution for embryos to be processed for PARP-1 immunostaining. Embryos were incubated in primary antibody solution for 1 h, washed vigorously in a series of fresh blocking solution (1 × 10 min, 1 × 30 min, and 1 × 10 min), and incubated for 1 h in secondary antibody; washes in blocking solution were repeated. Incubation with primary and secondary antibodies was done at 37°C or at room temperature for fluorescent detection of γH2AX or PARP-1, respectively. DNA was stained with 10 μg/ml 4’, 6-diamidino-2-phenylindole (DAPI) for 30 min, and embryos were washed and mounted in 3 μl of Vectashield antibleaching mounting medium (Vector Laboratories) on premarked slides.

Immunocytochemistry. Rat zygotes, sired by saline- or cyclophosphamide-treated males, were manipulated in parallel using previously described indirect immunofluorescence techniques with minor modifications (Barton et al., 2005). All embryo incubations were done at room temperature unless
was used for statistical analysis. γH2AX focal volume was assessed using the Isosurface module that creates a computer-generated representation of specified real volume objects within a data set; a threshold limit of 65 was used. Precise measurements and detailed statistical data derived from each object were exported into Excel-readable files for further categorization and statistical analysis.

**Quantification of PARP-1.** Single optical images representing characteristic staining patterns for each of the maternal and paternal pronuclei were imported as monochrome eight-bit TIFF files for quantitative analysis using MCID 7.0 imaging software (Imaging Research, St Catherines, Ontario, Canada). Optimal threshold limits for intensity and saturation were reserved across treatments. The density of PARP-1 immunofluorescence was measured as proportional grain area, defined as grain area/scan area. For each zygote, the perimeter of the male and female pronucleus was manually outlined to determine distinct scanning areas used for grain ratio calculations. Circles with a calibrated dimension of 20 × 20 µm, generating a scan area of 313.959 µm², were placed adjacent to each parental pronucleus to obtain background measures. Proportional grain area per pronucleus was adjusted to account for background immunofluorescence in all cases.

**Statistical analysis.** Student’s t-tests or Mann-Whitney rank sum tests were used to compare the γH2AX parameters and PARP-1 proportional grain areas of zygotic pronuclei from control animals with those that were sired by males chronically exposed to cyclophosphamide (p < 0.05). Error bars represent the mean ± SEM. SIGMASTAT 2.03 software package (SPSS, Chicago, IL) was used for all statistical analyses.

**RESULTS**

**Characterization of γH2AX Immunoreactivity**

**Postfertilization in the Absence of Genotoxic Stress**

Nucleosomal architecture and chromatin remodeling of the parental genomes are markedly different in the newly fertilized oocyte (Mclay and Clarke, 2003). Early postfertilization (PN1 and 2), the maternal chromatin was extensively stained with dense γH2AX fluorescence. The paternal genome displayed interconnecting amorphous clusters of various sizes throughout the chromatin at PN1; progression to PN2 resulted in the reorganization of γH2AX staining into multiple uniform punctate foci (Figs. 1a and 1b). At both pronuclear stages, γH2AX volume within the maternal chromatin was significantly greater than observed for the paternal genome (PN1, p < 0.001; PN2, p = 0.022) (Fig. 2A). Remarkably, at PN3, the numbers of γH2AX foci were dramatically reduced in both parental pronuclei (Fig. 1c) in zygotes fertilized by control males; male and female pronuclei remained consistently depleted of foci through PN4 and 5 (Figs. 1d and 1e). γH2AX appears to play a prominent role in the complex process of chromatin remodeling of parental genomes immediately after fertilization, as well as having a possible role in the silencing of the maternal genome during G1 of the zygotic cell cycle.

**Biphasic γH2AX Response in the Male Genome after Paternal Cyclophosphamide Exposure**

After fertilization, at PN1, the male pronucleus of embryos sired by cyclophosphamide-exposed fathers displayed enhanced levels of γH2AX fluorescence (Fig. 1f); at PN2, staining was markedly reduced to small foci, comparable to those observed in zygotes fertilized by spermatozoa from control males (Fig. 1g). Interestingly, at PN3, γH2AX fluorescence reemerged in the male pronucleus of zygotes fertilized by cyclophosphamide-exposed spermatozoa and continued to increase dramatically as zygotic development...
progressed through PN4 and 5 (Figs. 1h–j). Quantitative analysis confirmed the biphasic response of the paternal genome to DNA damage. The volume of \( \gamma \mathrm{H2AX} \) foci within the male pronuclei of embryos sired by cyclophosphamide-treated fathers was significantly increased at PN1 (\( p=0.013 \)), corresponding to phase 1, but it was not different from controls at PN2. This was followed by a progressive elevation through S phase (PN3 and 4) into G2 (PN5). This second phase represents a selective response of the zygote to paternal transmission of DNA damage (Fig. 2A). The maternal genome of zygotes fertilized by cyclophosphamide-exposed spermatozoa was not significantly different from controls (Figs. 1f–j and 2A). In conjunction with observations from embryos fertilized by control males, this demonstrates an initial requirement for \( \gamma \mathrm{H2AX} \) phosphorylation regardless of the presence of paternal genomic damage and highlights the absence of a \( \gamma \mathrm{H2AX} \) response in the intact maternal pronucleus of zygotes sired by cyclophosphamide-treated males.

**Number and Pronuclear Distribution of \( \gamma \mathrm{H2AX} \) Foci in Zygotes Fertilized by Cyclophosphamide-Exposed Spermatozoa**

Quantitative analysis of spots further revealed the dramatic induction of \( \gamma \mathrm{H2AX} \) foci after paternal cyclophosphamide treatment. The numbers of \( \gamma \mathrm{H2AX} \) foci were significantly higher in parental pronuclei of zygotes fertilized by cyclophosphamide-exposed spermatozoa at PN1 (\( p=0.009 \)) and increased dramatically at PN3 (\( p<0.001 \)), PN4 (\( p<0.001 \)), and PN5 (\( p=0.018 \)) compared to corresponding controls. In maternal pronuclei of embryos sired by cyclophosphamide-exposed males, the numbers of \( \gamma \mathrm{H2AX} \) foci were consistently low throughout zygotic development, similar to both parental genomes in controls (Fig. 2B).

Two distinct \( \gamma \mathrm{H2AX} \) focal populations have been described in normal and irradiated mammalian cells (McManus and Hendzel, 2005). To determine whether paternally mediated DNA damage induced distinguishable populations of \( \gamma \mathrm{H2AX} \) foci in the zygote, we characterized the volume distribution of \( \gamma \mathrm{H2AX} \) foci in embryos sired by cyclophosphamide-treated and control males (Fig. 3). In parental pronuclei of zygotes fertilized by control males, the majority of \( \gamma \mathrm{H2AX} \) foci were

![FIG. 2.](image)

A) The focal volumes of \( \gamma \mathrm{H2AX} \) were significantly increased in a biphasic pattern in paternal pronuclei of zygotes sired by males chronically exposed to cyclophosphamide. (B) The numbers of \( \gamma \mathrm{H2AX} \) foci were significantly elevated in paternal pronuclei of zygotes fertilized by cyclophosphamide-exposed males. The number of males in each treatment group was as follows: saline, \( n=6 \) and cyclophosphamide, \( n=6 \). The number of embryos analyzed at each pronuclear stage, representative of the six replicates, was as follows: saline, \( n=5, 11, 37, 28, \) and 5 for PN1, 2, 3, 4, and 5, respectively; cyclophosphamide, \( n=7, 7, 43, 23, \) and 7 for PN1, 2, 3, 4, and 5, respectively. Crosshatched bars, saline male pronucleus; black bars, cyclophosphamide male pronucleus; white bars, saline female pronucleus; and gray bars, cyclophosphamide female pronucleus. *\( p<0.02 \), **\( p<0.001 \).

![FIG. 3.](image)

The focal volume categories 1 and 2 of \( \gamma \mathrm{H2AX} \) account for the majority of foci induction in parental pronuclei of zygotes fertilized by saline- or cyclophosphamide-exposed males; populations of larger \( \gamma \mathrm{H2AX} \) foci were increased following paternal cyclophosphamide exposure. Stacked bar graph demonstrates the proportional contribution of each focal volume size category obtained from six replicates. Gray bars, category 1, \( 0.2-0.99 \mu m^3 \); speckled bars, category 2, \( 1.0-4.99 \mu m^3 \); white bars, category 3, \( 5.0-9.99 \mu m^3 \); and black bars, category 4, \( > 10.0 \mu m^3 \).
classified as category 1 or 2, with focal volumes between 0.2–
0.99 and 1.0–4.99 μm³, respectively. Interestingly, at PN1,
male pronuclei in embryos fertilized by cyclophosphamide-
treated spermatozoa displayed significantly more foci in
volume categories 1 (p = 0.014) and 3 (5.0–9.99 μm³; p = 0.001); at PN3, the numbers of γH2AX foci in categories
2 (p = < 0.001) and 3 (p = 0.002) were elevated, while at
PN4, categories 1 (p = 0.004), 2 (p = 0.001), and 3 (p = 0.016) were all increased significantly compared to control embryos at corresponding pronuclear stages. Preconceptional paternal cyclophosphamide treatment had no effect on the kinetics of γH2AX focal volumes in maternal pronuclei. These data demonstrate that DNA damage imparted to the embryo via the spermatozoon causes an increase in the population of larger γH2AX foci in addition to the accumulation of smaller foci.

To further characterize the γH2AX damage response in the zygote, we qualitatively assessed 3D focal distribution within the male and female pronuclei. As indicated above, the female chromatin at PN1 and 2 was highly stained by γH2AX (see Supplementary Movies 1 and 2, supporting information); at PN3, 4, and 5, female pronuclear staining was dramatically reduced to a single focus located at the perimeter, occasionally accompanied by sparse small foci situated randomly throughout the chromatin (see Supplementary Movies 3 and 4, supporting information). At PN2, γH2AX foci were dispersed evenly throughout the male pronuclei of zygotes sired by control and cyclophosphamide-exposed fathers. However, in contrast to the small number of foci distributed intermittently throughout the paternal pronucleus of controls from PN3 through 5 (Supplementary Movie 3), zygotes sired by cyclophosphamide-exposed males displayed γH2AX clusters concentrated in the perinucleolar regions as well as throughout the pronucleus (Supplementary Movie 4). Therefore, γH2AX foci are distributed throughout the entire male pronucleus following paternal cyclophosphamide treatment.

**Paternal Cyclophosphamide Exposure Activates Poly(ADP-ribosylation during Zygotic Development**

We investigated PARP-1 induction in the zygote following chronic paternal cyclophosphamide exposure to determine the competence of the early embryo to activate signaling networks with known involvement in the repair of DNA lesions. Immediately after fertilization, both haploid pronuclei displayed minimal PARP-1 immunofluorescence staining in embryos sired by saline- and cyclophosphamide-exposed males (Figs. 4a and 4e). In controls, male and female pronuclei maintained baseline levels of staining from PN3 through 5 (Figs. 4b–d). Intriguingly, in zygotes sired by cyclophosphamide-treated fathers, PARP-1 immunofluorescence was dramatically elevated at PN3 in both parental genomes and remained intense through zygotic development, coincident with the second phase of γH2AX foci induction (Figs. 4f–h). Furthermore, a large proportion of male and female pronuclei in zygotes at PN3–5 displayed dense PARP-1 staining in perinucleolar regions (Figs. 4f–h), compared to controls. Quantitative analysis confirmed that PARP-1 proportional grain areas of male and female pronuclei were significantly increased at PN3 (p = < 0.001) and 4 (p ≤ 0.005) and maintained a hyperactivated state at PN5, compared to controls (Fig. 5). Thus, fertilization by spermatozoa chronically exposed to a genotoxic agent rapidly triggered DNA damage response.
bioindicators in both parental genomes, further emphasizing the importance of zygotic pronuclear cross talk in the maintenance of genomic stability.

**DISCUSSION**

The phosphorylation of H2AX has specialized physiological functions beyond DNA damage detection and repair in the zygote. Prior to fertilization, the metaphase II chromosomes of the dormant oocyte are extensively labeled with γH2AX (Derijck et al., 2006). Fertilization initiates a multistage process of chromatin remodeling of both gametes; oocyte chromatin with meiotic features is transformed to strictly mitotic chromatin, and highly compacted sperm chromatin is decondensed to produce a transcriptionally competent paternal genome (Mclay and Clarke, 2003). Interestingly, DNA damage–independent γH2AX foci have been described in mitotic cells (McManus and Hendzel, 2005), confirming a biological role for γH2AX in meiotic and mitotic chromosomes. During G1 (PN1 and 2) of the first cell cycle, inactive maternal chromatin, that is poorly accessible to transcription factors (Spinaci et al., 2004), was highly stained (Fig. 1, Derijck et al., 2006); paternal chromatin undergoing protamine-histone exchange and elaborate remodeling displayed large amorphous clusters in PN1 that were rapidly reduced to punctate foci in PN2. Intriguingly, γH2AX was remarkably depleted in both pronuclei at S phase (Fig. 1), corresponding to the onset of replication and transcription in the one-cell embryo; DNA synthesis in the male pronucleus precedes that in the female (Ferreira and Carmo-Fonseca, 1997). We propose that H2AX phosphorylation is involved in the control of chromatin structural reorganization, ensuring proper temporal execution of early zygotic events.

Exogenous genotoxic insults induce phosphorylation of H2AX over megabases of chromatin, tethering each double-strand break (Kruhlak et al., 2006). Alterations in spermatozoal genomic integrity, acquired during 4 weeks of preconceptional exposure to cyclophosphamide, were detected effectively in the early embryo. Intriguingly, γH2AX emerged in two distinct phases in the paternal genome of zygotes sired by cyclophosphamide-treated males, each corresponding to important regulatory processes of the first zygotic cell cycle. The initial spike of γH2AX (Fig. 2A) occurred in the recondensing male pronucleus (PN1) during nucleoprotein exchange (Mclay and Clarke, 2003). The replacement of protamines by histones is a phosphorylation-sensitive process, emphasizing a dual role for γH2AX in chromatin remodeling and DNA damage recognition in the early male pronucleus. Zygotes sired by cyclophosphamide-exposed males were hyperacetylated at histone H4 as early as G1 of the first cell cycle (Barton et al., 2005). Histone acetylation regulates H2AX phosphorylation (Park et al., 2003) which is required for the recruitment of additional histone-acetyltransferase complexes to sites of DNA double-strand breaks (Murr et al., 2006). Alkylation of sperm DNA and nuclear proteins by cyclophosphamide may distort nuclear compaction, thereby generating an abnormally permissive chromatin conformation, permitting enhanced accessability to chromatin-modifying proteins and DNA repair factors. Immediately after fertilization, histone hyperacetylation and H2AX phosphorylation may cooperate to facilitate chromatin reconfiguration at lesion sites in zygotes with paternal DNA damage, thereby triggering a DNA damage histone code that mediates events implemental for DNA damage resolution beginning in S phase (Murr et al., 2006; Park et al., 2003).

DNA double-strand breaks initiate local chromatin expansion independent of γH2AX. However, consistent with our previous observation of increased chromatin dispersion from PN3 through 5 in zygotes fertilized by cyclophosphamide-exposed spermatozoa (Barton et al., 2005), γH2AX may be required for sustained chromatin relaxation, thus augmenting accessibility at DNA damage sites (Kruhlak et al., 2006) in the male pronucleus during the second γH2AX induction phase (Fig. 2A). Cyclophosphamide-induced hyperacetylation and hypomethylation of the male pronucleus further supports the existence of a highly permissive chromatin state (Barton et al., 2005). γH2AX is dispensable for the initial recruitment of signaling and DNA repair proteins; however, repair factor concentration, assembly and stabilization at double-strand break sites are dependent on H2AX phosphorylation (Celeste et al., 2003). The striking increase in γH2AX focal volume and

**FIG. 5.** Quantitative analysis of PARP-1 proportional grain area. Maternal and paternal pronuclear PARP-1 staining was significantly increased at PN3 and 4 and remained hyperactivated at PN5 in zygotes sired by cyclophosphamide-treated rats. The number of males in each treatment group was as follows: saline, n = 4 and cyclophosphamide, n = 4. The number of embryos analyzed at each pronuclear stage, representative of the four replicates, was as follows: saline, n = 10, 16, 11, and 2 for PN1 and 2, 3, 4, and 5, respectively; cyclophosphamide, n = 3, 28, 11, and 3 for PN1 and 2, 3, 4, and 5, respectively. Crosshatched bars, saline male pronucleus; black bars, cyclophosphamide male pronucleus; white bars, saline female pronucleus; and gray bars, cyclophosphamide female pronucleus. *p < 0.01.
numbers (Figs. 2A and 2B) in PN3–5 in response to paternal cyclophosphamide exposure may function to recruit and maintain the localization of numerous repair factors to the vicinity of the damage, assisting in the processing of the DNA lesions. The accumulation of a higher concentration of repair proteins may further affect chromatin structure and alleviate transcriptional repression during the repair process.

Large-scale genomic repositioning does not occur as a result of DNA double-strand breaks; therefore, an increasing focal size has been suggested to correlate with γH2AX spreading over a large chromatin domain, providing additional binding sites for DNA damage response proteins (Kruhlak et al., 2006). Zygotes fertilized by spermatozoa chronically exposed to cyclophosphamide displayed an increased number of larger foci, beginning in S phase through G2 (Fig. 3). These late-arising larger foci may represent complex lesion repair and/or foci, spreading over a large chromatin domain, providing additional binding sites for DNA damage response proteins (Kruhlak et al., 2006). Thus, cyclophosphamide-induced DNA damage in the spermatozoal genome activates distinct yet interrelated pathways involved in the detection and signaling of male-mediated genomic lesions. The enhanced presence of γH2AX and PARP-1 at G2 in embryos sired by males exposed to a genotoxicant indicates that the resolution of paternally mediated DNA damage is incomplete prior to the first cleavage division. Sophisticated mechanisms for the complete resection of DNA lesions are required in later cleavage stages to ensure the faithful transmission of genomic sequence information required for successful embryogenesis and the generation of healthy offspring.

SUPPLEMENTARY DATA

Supplementary Movies 1–4 are available online at http://toxsci.oxfordjournals.org/.

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