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Andrology

Effect of Pentoxifylline on Tumor Suppressor and Proto-Oncogene Apoptosis in Sperm

David T. Maxwell,[1] John D. Jacobson,[1] Alan King,[1] and Philip J. Chan[1,2,3]

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***Purpose*:** Pentoxifylline (PTX), a methylxanthine phosphodiesterase inhibitor reduces superoxide anions responsible for DNA apoptosis. The null hypothesis was that PTX was equally effective in reducing damage to specific cell genes. The objective was to determine the DNA integrity of the *BRCA1* tumor suppressor gene and the c-*myc* proto-oncogene after PTX.
***Methods*:** Sperm (64 samples, 4 patients) were preincubated in either 0 (control) or 3.6 mM PTX (30 min), washed and incubated for 4 h at either 37 or 40°C heat shock activation. Single primer polymerase chain reactions (PCR) were carried out on lysed sperm targeting either *BRCA1* exon 11 or c-*myc* exon 1. Control single-stranded DNA (ssDNA) were stained with 9 μM Hoechst 33342 (blue) while PTX-treated ssDNA were stained with SYBR Gold (green). Nytran membrane discs with control ssDNA were hybridized to PTX-derived ssDNA. Fluorescent images stored in a microarray design were analyzed using ANOVA and Students' *t*-test for ($P < 0.05$) significance.
***Results*:** *BRCA1* integrity was higher with PTX pretreatment (93.3 + 10.4 vs. control 50.5 + 9.2; mean + SEM). In contrast, there was no difference in c-*myc* integrity (56.8 + 9.0 vs. 41.7 + 6.4). Sense or antisense primers gave similar DNA fragmentation results.
***Conclusions*:** The data showed PTX pretreatment protected *BRCA1* but not c-*myc* suggesting that PTX did not equally protect different cell genes. A possible explanation was that proto-oncogenes had more fragile sites. The study involved the DNA disc chip assay to assess separate PCR-amplified sense and antisense strands. The results suggested that both strands were equally affected by PTX pretreatment.

KEY WORDS: Comparative genomic hybridization; pentoxifylline; proto-oncogene; spermatozoa; tumor suppressor gene.

INTRODUCTION

Pentoxifylline (PTX), a methylxanthine phosphodiesterase inhibitor, increases cyclic adenosine 3′:5′ monophosphate (cAMP) intracellularly (1,2), reduces superoxide anions and inhibit tumor-necrosis factor—alpha (TNF-alpha) responsible for DNA fragmentation and apoptosis or programmed cell death (3–7). PTX has been used to stimulate sperm motility and improve fertilization (2). Furthermore, PTX has been used in clinical procedures to scavenge reactive oxygen species and reduce lipid peroxidation associated sperm membrane damage and DNA apoptosis (4,8–10). While most studies focus on the assessment of the entire genome for DNA damage, it is uncertain if the integrity of each specific gene is equally affected by PTX-mediated inhibition of apoptosis. The null hypothesis was that PTX was equally effective in reducing damage to specific cell genes. The objective was to determine the DNA

[1] Department of Gynecology and Obstetrics, Loma Linda University School of Medicine, Loma Linda, California.
[2] Department of Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda, California.
[3] To whom correspondence should be addressed at Loma Linda University Center for Fertility and In Vitro Fertilization, 11370 Anderson Street, Suite 3950, Loma Linda, California 92354; e-mail: pchann@yahoo.com.

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integrity of the *BRCA1* tumor suppressor gene and the c-*myc* proto-oncogene in sperm after PTX pretreatment and followed by heat shock activation. The information obtained would help to stimulate further studies examining the differential effect of chemical agents on the integrity of different genes.

MATERIALS AND METHODS

Specimen Preparation

The sperm cells were from male partners of infertile couples attending an assisted reproductive technologies program. The spermiograms of the male patients were considered normal with normal concentration, total motility and normal morphology as assessed through basic semen analyses. Data from patients that involved epididymal sperm aspiration or donor sperm were not included in the study. A total of 64 washed specimens from four cases were evaluated. This study that was based on the multiple cell comet assay was approved by the Loma Linda University Institutional Review Board.

Each sperm specimen was divided into two portions and washed using either the swim-up (control) or pentoxifylline wash procedure (11). The swim-up procedure consisted of layering a 1 mL volume of HEPES-based human tubal fluid culture medium with 5% human serum albumin (mHTF, Irvine Scientific, Santa Ana, CA) over on top of 1 mL of semen in a centrifuge tube. The tubes were placed in a 37°C waterbath for 30 min and the bottom layer of semen pipetted out and disposed. The remaining tubes of culture medium containing the motile sperm were centrifuged (300*g*, 10 min), resuspended in 0.4 mL mHTF and labeled as the swim-up group.

The pentoxifylline wash procedure involved mixing 1 mL of each semen specimen with 1 mL of pentoxifylline (Sigma Chemicals, St. Louis, MO) dissolved in mHTF medium to obtain a 3.6 mM final concentration. Each mixture was incubated for 30 min at 37°C in air before being layered over the 90:45% silane colloid layers (Isolate, Irvine Scientific, Santa Ana, CA) in a centrifuge tube and centrifuged at 300*g* for 10 min (11). The bottom 90% layer was removed, mixed with an equal volume of mHTF medium and centrifuged (300*g*, 10 min). Each pellet of sperm was resuspended in 0.4 mL mHTF medium and labeled as the pentoxifylline group.

A mild heat shock treatment was applied to each tube of swim-up or pentoxifylline-treated sperm. This consisted of incubating the tubes of sperm at 40°C in air for 4 h (12). A second set of washed sperm was kept at 37°C in air for 4 h as the unheated control. After 4 h, the sperm cells were stored frozen at −30°C for several weeks and thawed for the DNA assays. The integrity of the tumor-suppressor gene, *BRCA1* and the proto-oncogene, c-*myc*, were assessed through a novel DNA disc chip assay based on comparative genomic hybridization (described below). These genes were selected based on the opposing functions of *BRCA1* and c-*myc* genes, and on the experience with polymerase chain reaction (PCR) primers for these genes at this laboratory.

The DNA Disc Chip Assay

The DNA disc chip assay procedure was as follows: The frozen heat-treated and control sperm cells were thawed at room temperature for 10 min. The sperm were centrifuged at 3000*g* for 1 min to remove the culture media. Each resultant pellet was resuspended in 4°C alkaline lysis buffer (1% *N*-Lauroylsarcosine, 1.0 M Tris–HCl, pH 7.5, 0.5 M EDTA, 0.3 M mercaptoethanol, pH adjusted to >10 with sodium hydroxide pellets) for 20 min with periodic vortexing to release the sperm DNA. The tubes of sperm DNA were then placed in a PCR thermal cycler and heated at 94°C for 5 min to denature the DNA into single strands similar to the "hot start" PCR protocol. Single-stranded DNA (ssDNA) were generated using the single primer PCR procedure. The PCR amplifications of sperm DNA involved either the sense or antisense primer targeting either the protein-coding region of *BRCA1* (13) at exon 11 (434 base pair, bp) or the regulatory region (14) of c-*myc* exon 1 (256 bp).

The DNA staining procedure was carried out in diffuse lighting or darkened room. The control (swim-up or pentoxifylline-treated at 37°C) sperm ssDNA (derived from sense or antisense primers) were stained in 9 μM bisbenzimide (Hoechst 33342, Sigma Chemicals, St. Louis, MO) for 5 min and centrifuge-washed twice to remove excess stain (3000*g* for 1 min). The ssDNA (derived from sense or antisense) from heat shocked sperm (swim-up or pentoxifylline-treated at 40°C) were stained in 1:10,000 diluted SYBR Gold stock stain (Molecular Probes, Eugene, OR) for 5 min and excess stain removed as recommended by the manufacturer. It was unnecessary to adjust DNA concentration in the tubes due to the matched control-test design and saturation of the positive-charged miniature disc.

The tiny round discs used in the DNA disc chip assay were made by pressing a 2 mm Acupunch biopsy

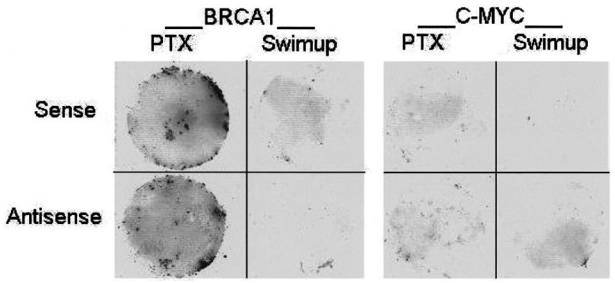

Fig. 1. A DNA disc chip assay showing bright fluorescent *BRCA1* exon 11 single-stranded DNA (ssDNA) of both sense and antisense strands in the hybridized membrane discs (2 mm diameter) associated with DNA integrity in sperm cells pretreated with 3.6 mM pentoxifylline (PTX) and heat shock treated at 40°C for 4 h. In contrast, the ssDNA of *BRCA1* after swimup processing (control) and the ssDNA of c-*myc* exon 1 of sperm exhibited low fluorescence suggesting DNA fragmentation and apoptosis. All control matching ssDNA embedded on the discs were analyzed and found to have similar fluorescence. Each digitized image was reduced by 80%, cut and pasted on to an image of a multiwell tray to form a microarray of images for storage, comparison, analysis, and presentation purposes.

punch pen (Acuderm, Ft. Lauderdale, FL) into the Nytran (Schleicher & Schuell, Keene, NH) membrane sheet and using a plastic stylet to expel the round discs. The size of the disc was chosen to fit the 40× magnification of the fluorescent microscope used in this lab. The discs could be attached into the wells of a microtiter plate to process greater volume of samples, similar to a microarray design. For this study, each disc was held using a pair of microforceps and first dipped into the tube of control (37°C) ssDNA (Hoechst 33342, blue fluorescence) for a few seconds and blotting off the excess. Then, the disc was dropped into the tube of test (40°C) ssDNA (Sybr Gold, green-golden fluorescence). This process was repeated several times to obtain replicated data points.

Each tube with submerged discs was allowed to hybridize for 10–20 s. Several discs were removed using a pair of microforceps, and lined up on a glass slide to form a DNA chip. Each glass slide with the DNA discs was examined using an ultraviolet (UV) epifluorescent microscope at 40× magnification. The fluorescent images were captured by placing an inexpensive QuickCam Pro camera (Logitech, Fremont, CA) over the microscope eyepiece and saving the 640 × 480 pixels images to hard disk (Fig. 1). For comparison purposes, the color images were computer-cut and pasted on to a photo of a 50-well microtiter plate in a microarray design using the Microsoft Paint program. The images were converted to grey-scale and the pixel intensity of each disc analyzed using Paint Shop Pro 6 software (Jasc Software, Eden Prairie, MN). Greater damage of ssDNA was observed as a lower pixel intensity (green-golden fluorescent) of the disc due to lack of pairing or mismatch with the control ssDNA.

Statistical Analysis

The pixel intensity of each fluorescent disc containing hybridized DNA was obtained from the computer imaging software and expressed as mean ± SEM (standard error of the mean). The pixel intensity of each disc from heat-treated (40°C) sperm DNA were matched or paired with the pixel intensity of corresponding internal calibration control (37°C) sperm. Analysis of variance (ANOVA) followed by significance testing of the means using the Students' *t*-test statistic were used. A value of $P < 0.05$ was considered significant.

RESULTS

The sperm specimens used for the present study (Table I) were considered normal in accordance with the values set by the 1999 fourth edition of the World Health Organization (WHO) Semen Analysis Manual (15). There were no remarkable differences between the postwash motilities of the control swim-up from semen group and the PTX pretreated group.

The integrity of the *BRCA1* gene was almost twofold greater ($P < 0.05$) in sperm with PTX pretreatment (93.3 ± 10.4 vs. 50.5 ± 9.2) when compared with control sperm (Table II). In contrast, c-*myc* (56.8 ± 9.0 vs. 41.7 ± 6.4) gene integrity was similar for both the PTX pretreatment and control groups. Interestingly, the antisense strand of *BRCA1* appeared to be less stable than the sense strand while the opposite was observed for the c-*myc* gene. Statistically, the sense and antisense data were considered similar in terms of intensity of DNA fragmentation. Blank Nytran membrane discs were used in

Table I. Basic Sperm Parameters and Motilities After Processing Through Either the Swim-Up from Semen (Control) or Pentoxifylline (3.6 mM) Treatment

Parameter	Results (mean ± SEM)	WHO normal reference (15)
Concentration (million/mL)	49.3 ± 1.0	>20 million/mL
Total motility (%)	62.8 ± 5.2	>50% motile
Strict normal morphology (%)	11.3 ± 1.8	>4% normal
Postwash total motility		
Swim-up from semen (%)	82.3 ± 7.5	
Pentoxifylline treatment (%)	85.5 ± 4.4	

Table II. A Comparative Genomic Hybridization DNA Disc Chip Assay of the Tumor Suppressor Gene, *BRCA1*, and the Proto-Oncogene, c-*myc*, in Sperm Cells with and Without Pentoxifylline (3.6 mM) Pretreatment

		Pixel intensities of fluorescent discs (mean + SEM)		
Treatment	*N*	Antisense ssDNA of heated sperm paired with control sense ssDNA on disc	Sense ssDNA of heated sperm paired with control antisense ssDNA on disc	Cumulative data of heated ssDNA paired with control ssDNA
BRCA1 exon 11				
Swim-up control	8	50.5 ± 18.7	50.0 ± 10.7	50.5 ± 9.2
Pentoxifylline	8	86.5 ± 8.8[a]	100.1 ± 21.8[a]	93.3 ± 10.4[a]
Negative control	4	n/a	n/a	25.3 ± 1.5
c-*myc* exon 1				
Swim-up control	8	47.0 ± 11.5	34.7 ± 11.8	41.7 ± 6.4
Pentoxifylline	8	71.0 ± 14.0	42.6 ± 8.2	56.8 ± 9.0
Negative swim-up	4	n/a	n/a	25.6 ± 3.0

[a] Different from control. $P < 0.05$.

the determination of background fluorescence for the negative controls and did not have sense or antisense data.

DISCUSSION

The methylxanthine phosphodiesterase inhibitor, PTX [1-9[5-oxohexyl]-3-7-dimethylxanthine), has been reported to stimulate sperm motility (1,2) by increasing intracellular cyclic adenosine 3′:5′ monophosphate (cAMP) and by reducing superoxide anion and reactive oxygen species (ROS), which damage DNA (9,10). Pretreatment of asthenozoospermic or slow moving sperm with PTX has been shown to improve in vitro fertilization of oocytes (2). Consistent with the known protective effect of PTX on DNA, sperm pretreated with PTX also showed less DNA damage in specific genes after heat shock treatment. Specifically, the tumor suppressor gene, *BRCA1*, remained relatively intact in PTX pretreated sperm when compared with untreated sperm.

However, the proto-oncogene, c-*myc*, was not protected by PTX pretreatment. The results suggested that PTX did not act with equal efficacy in protecting different genes, in this case, genes involved with cell proliferation and growth. One possible explanation was that proto-oncogenes might have more fragile sites that are prone to damage (16) or that the DNA repair mechanisms are absent or slow reacting for proto-oncogenes. The c-*myc* proto-oncogene is located on the long arm of Chromosome 8. The proto-oncogenes include c-*myc*, c-*fos* and c-*jun*, and are nuclear proto-oncogenes involved in sperm function (17), and mediate the action of growth factors during cell proliferation (18). The proto-oncogenes code for nuclear proteins that regulate DNA replication and RNA transcription during increased cell proliferation and differentiation (19). Tumor suppressor genes such as *BRCA1* (20–26) oppose the proliferation and growth directives of proto-oncogenes. The *BRCA1* gene is located on the long arm of Chromosome 17 and mutations of this gene are associated with breast, ovarian, prostate, and colon cancers (27).

The mechanism of PTX action involves inhibiting apoptosis or programmed cell death characterized by DNA fragmentation (28). PTX has been reported to interfere with lipid signaling of cytokines and inhibit tumor-necrosis factor—alpha (TNF-alpha) which is linked to the Caspase 3 enzyme for apoptosis (6). However, the results showing differences in PTX action on DNA integrity depending on gene type suggest an alternate apoptotic pathway unaffected by PTX. The differential protective effect of PTX on specific genes is important and suggest that apoptosis studies that focus on selected genes may miss important DNA alterations in other parts of the genome.

In this study, the DNA disc chip assay was utilized to assess the integrity of the DNA fragments produced by PCR amplification. The assay was simple and cost-effective for gene analysis and permitted small laboratories to take part in the exciting fields of comparative genomic hybridization (29) and microarray analysis (30). The principle of this fluorescent assay was based on undamaged test DNA hybridizing with control DNA located on a membrane disc similar to the Southern blot (31). Intact test DNA exhibits maximal fluorescence in contrast to damaged or mutated test DNA that fails to hybridize efficiently and thus exhibit reduced fluorescence. Drawbacks of the assay included the requirement of a fluorescent microscope, a computer, and a digital camera. More studies

are needed to expand the capabilities of the assay to include RNA expression analysis.

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