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SHORT COMMUNICATION

Flow cytometric characterization of apoptosis and chromatin damage in spermatozoa

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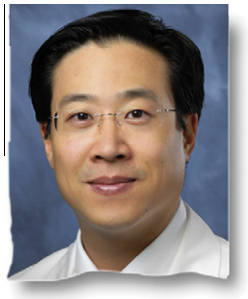

Howard Kim, MD is a board-certified urologist with Cedars-Sinai Medical Group. He graduated with distinction from Stanford University and received his medical doctorate from the University of California, San Francisco, where he was President of the Alpha Omega Alpha National Medical Honor Society. He completed his general surgery internship and urology residency at Massachusetts General Hospital and Harvard Medical School. He then completed a fellowship at the Center for Male Reproductive Medicine at Weill Cornell Medical College. Dr. Kim's clinical interests include general urology, andrology, male infertility and erectile and sexual dysfunction.

Abstract Apoptosis has been implicated in sperm chromatin damage; it is unclear whether apoptosis occurs through cytoplasmic or mitochondrial pathways. Sperm has minimal volume of cytoplasm but prominent mitochondria. Propidium iodide (PI), annexin V (AV), $DiIC_1(5)$ and proprietary fluorochrome (PF-1) were used to investigate apoptosis activation in human sperm using multichannel flow cytometry. There was a time-dependent increase in staining of spermatozoa with both AV and PF-1 and decrease in mitochondrial staining with $DiIC_1(5)$. These results strongly suggest that the drop in mitochondrial potential precedes changes in membrane phospholipids, and thus suggest apoptotic activation through mitochondrial pathway in human spermatozoa.

KEYWORDS: apoptosis, chromatin damage, flow cytometry, spermatozoa

Introduction

There is increasing interest in the prognostic value of DNA integrity assays in human reproduction and toxicology. Sperm chromatin damage is associated with increased risk of spontaneous abortions and impaired embryo development (Zini et al., 2011). Apoptosis has been implicated as a primary mechanism for sperm chromatin damage (Sakkas et al., 2004).

Apoptosis is a complex cascade of sequential, enzymically regulated events leading to controlled cell death associated with chromatin fragmentation and clumping. Nuclear condensation and endonuclease-mediated DNA fragmentation are essential steps in apoptotic cell death (Petit et al., 1997). The precise pathways of apoptosis in spermatozoa are poorly understood. The aim of this study was to investigate whether induction of apoptosis using the classic somatic cell inducer (nitroprusside sodium) activates sperm apoptosis through a

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mitochondrial as opposed to a cytoplasmic pathway. Spermatozoa have minimal cytoplasmic volume, thus it was postulated that sperm chromatin damage is most likely the result of the mitochondrial and not cytoplasmic receptor activation mechanism. Accumulating evidence points to the major role of mitochondrial membrane permeabilization in mammalian cell apoptosis (Estaquier et al., 2012). A better understanding of how apoptosis leads to sperm chromatin damage may enable development of new diagnostic and therapeutic options for infertile men.

This study examined the apoptotic pathway in spermatozoa using flow cytometry with propidium iodide (PI), annexin V (AV) and proprietary fluorochrome (PF-1). These fluorochromes are known to be effective in staining dead cells and apoptotic cells. $DiIC_1(5)$ was used to measure mitochondrial membrane permeabilization, an early step in apoptosis induction.

Materials and methods

The study was carried out under Institutional Review Board approval (Granted 28 November 2007) with patient consent.

Preparation of semen specimens

The discarded portion of ejaculated semen was purified using Percoll gradient to select the most viable and motile intact spermatozoa. Spermatozoa were counted using a haemocytometer. Aliquots of 1×10^6 spermatozoa/ml were prepared from each subject.

Induction of apoptosis

Half of each specimen was incubated for 1 h and 2 h in 10^{-4} mol/l sodium nitroprusside solution (Wu et al., 2004).

Fluorochromes

Semen samples of seven men were used to optimize assay conditions for PI, AV, $DiIC_1(5)$, and PF-1 (Invitrogen, Carlsbad, CA, USA). Dead spermatozoa are permeable to PI; dead or apoptotic cells are permeable to PF-1. Intact cells are impermeable to both. AV binds to phosphatidylserine on the inner side of cell membranes during apoptosis and indicates late apoptotic events. Overall, 160 flow cytometric analyses were performed in order to establish the optimal analytical settings for multicolour sperm fluorescence-activated cell sorting (FACS) and to assure a high level of reproducibility. $DiIC_1(5)$ is a marker for polarized mitochondrial membrane permeabilization in healthy cells and has reduced signal in apoptotic cells with depolarized mitochondrial membranes. One microliter of fluorochrome was added to each sample containing 1×10^6 spermatozoa/ml.

Fluorescence-activated cell sorting

All samples were analysed using BD FACSVantage (BD Biosciences, San Jose, CA, USA). Forward and side scatter gates were used to identify spermatozoa. Populations of intact, apoptotic and dead spermatozoa before and after apoptosis induction were analysed using multicolour flow cytometry. Results of 237 FACS runs were analysed; 160 were used for assay development and optimization and 77 for actual analysis.

Activation of apoptosis via the mitochondrial pathway

Ejaculated sperm specimens were stained with PI, AV and $DiIC_1(5)$ and analysed with flow cytometry. Specimens were analysed at two time points, 1 h and 2 h after apoptosis induction, to determine the relative activities of fluorochromes. The percentages of spermatozoa stained with specific markers before and after induction of apoptosis were compared. Statistical significance was determined by the chi-squared test performed on entire data set from all FACS runs.

Results

The relationship of induced apoptosis with sperm chromatin damage

For non-induced spermatozoa, 471×10^3 spermatozoa (0.5%) were positive for AV and 7820×10^3 spermatozoa (7.8%) were positive for PF-1 and PI. After apoptosis induction, 1190×10^3 (1.2%) were positive for AV and 10.7% were positive for PF-1 and PI. The difference was statistically significant for both AV and PF-1/PI staining ($P < 0.0001$), confirming that sodium nitroprusside induces classic apoptotic changes in ejaculated spermatozoa.

Activation of apoptosis via the mitochondrial pathway

Of all gated spermatozoa analysed with flow cytometry, at 1 h 36.3% were AV positive and 37.5% were $DiIC_1(5)$ positive in non-induced group, and 45.7% were AV positive and 33.9% were $DiIC_1(5)$ positive in induced group. Both the increase in AV signal (apoptosis marker) and the decrease in $DiIC_1(5)$ (activation of mitochondria) after apoptosis induction were statistically significant ($P < 0.0001$) (**Figure 1**). At 2 h after induction, AV binding was 35.5% in the non-induced spermatozoa and 48.8% in induced spermatozoa ($P < 0.0001$). AV staining increased 1.41-fold (from 9.4% to 13.3%) and $DiIC_1(5)$ decreased 4.4-fold (from 4.4% to 1.0%) at 1 h versus 2 h after induction, indicating that most changes in $DiIC_1(5)$ staining occurred during the first hour of apoptosis induction.

Discussion

This study demonstrated increased staining of spermatozoa with both AV and PF-1 after induction of apoptosis. AV is a well-documented marker for apoptosis. PF-1 is associated with TUNEL-positive spermatozoa with chromatin damage. This suggests that treatment of spermatozoa with a classic activator of apoptosis (sodium nitroprusside) leads to activation of the apoptotic pathway in spermatozoa. There was no increase in PF-1 signal in non-induced spermatozoa over 2 h, suggesting sperm chromatin damage is a consequence of active induction of apoptosis and not a spontaneously occurring, short-term event.

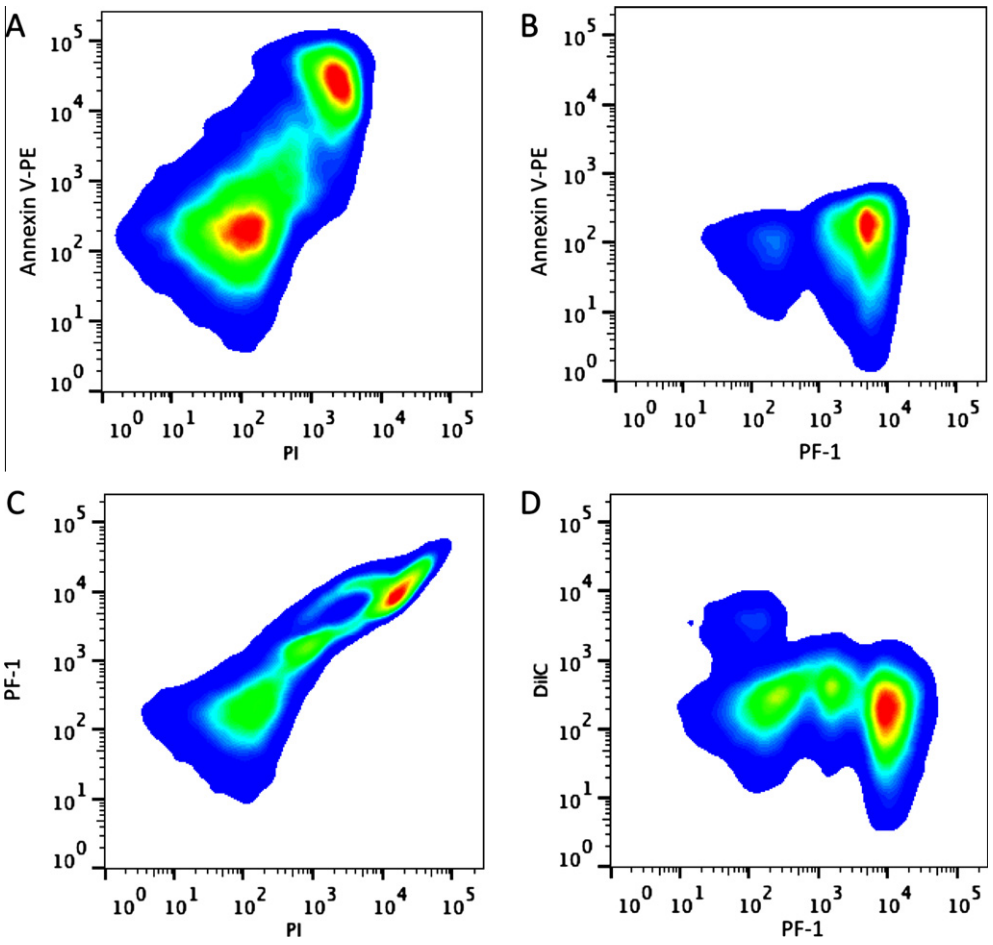

Figure 1 FACS analysis of human spermatozoa stained with different fluorescent markers. (A) With progression of the apoptosis signal, membrane is permeable to annexin V and PI; thus both signals increase in intensity. (B) AV is a late marker of apoptosis as spermatozoa with PF-1 fluorescence values between $0-10^4$ show no change in AV. (C) Typical fluorogram of PF-1 against PI shows broad range of PF-1 fluorescent intensity for PI negative spermatozoa; this indicates that some PI-negative spermatozoa are PF-1-positive, allowing early detection of apoptotic spermatozoa. (D) $DiIC_1(5)$ drops in intensity as PF-1 increases; in PF-1-negative spermatozoa $DiIC_1(5)$ has highest level of intensity (10^3-10^4). DiIC — DiIC1(5) stain; PF-1 = proprietary stain; PI = propidium iodide, Annexin V-PE - AV conjugated to phycoerythrin.

The observed decrease in $DiIC_1(5)$ staining suggests that sperm chromatin damage is a result of apoptosis induction through the mitochondrial pathway. The $DiIC_1(5)$ dye is a classic marker for polarized mitochondrial membrane permeabilization in somatic cells and has a reduced signal in apoptotic cells with depolarized mitochondrial membranes. There was a significant decrease in the number of spermatozoa that were $DiIC_1(5)$ positive, suggesting mitochondrial membrane damage. These cells were AV positive, confirming apoptosis. The time—response curve indicates that changes in mitochondrial potential precede changes in membrane phospholipids. This observation suggests that initiation of apoptosis in spermatozoa is mitochondrial in nature, rather than cytoplasmic. As far as is known, this phenomenon, although intuitive as spermatozoa have minimal cytoplasmic volume, has not been experimentally confirmed in ejaculated human spermatozoa to date. A comprehensive understanding of these processes may enable development of clinically relevant sperm DNA integrity assays and allow potential development of new therapeutic approaches that block mitochondrial activation of apoptosis.

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