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Andrology

Modified Isocratic Capillary Electrophoresis Detection of Cell-free DNA in Semen

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***Purpose*:** The objectives were: i) to analyze semen for the presence of cell-free DNA and ii) to determine the association between sperm parameters and cell-free DNA.
***Methods*:** Cell-free DNA in semen ($N = 25$ cases) were detected using the modified capillary gel electrophoresis (CE) procedure. SYBR-Gold was used to stain high (12 Kb) and low (1 Kb) molecular weight DNA fragments and the images analyzed.
***Results*:** The quantity of low-molecular weight cell-free DNA was positively correlated to rapid progression, curvilinear velocity (>40 μ/s), normal strict morphology and capacitation index. High-molecular weight cell-free DNA intensity index was negatively correlated to post-wash hyperactivation. Sperm concentration was not related to cell-free DNA quantity. The sperm freezing process did not increase cell-free DNA but reduced the more labile low-molecular weight cell-free DNA.
***Conclusions*:** Cell-free DNA present in semen was correlated to important sperm parameters linked to normal sperm function. The data suggested the possible use of cell-free DNA as a marker of semen quality. This study reports on the novel finding of cell-free DNA released along with sperm during each ejaculation.

KEY WORDS: Apoptosis and active secretion; circulating nucleic acids; infertility; spermatozoa.

INTRODUCTION

Cell-free DNA detected in the maternal circulation during pregnancy have been traced to fetal origins (1,2). Circulating cell-free DNA also appear in healthy individuals (3) and in cancer patients due to apoptosis (4) and active secretion (5). Information on the presence of cell-free DNA in the seminal plasma is lacking. Recent reports have emphasized the importance of studying the occurrence of cell-free DNA in vivo and the association with the disease state of the human body (3–5). The null hypothesis was that cell-free DNA was completely absent in ejaculated semen. The objectives were: i) to analyze semen for the presence of cell-free DNA and ii) to determine the association between sperm parameters and cell-free DNA. The capillary electrophoresis (CE) method (6–9) was modified to provide a rapid assay for cell-free DNA in semen.

MATERIALS AND METHODS

Processing of Semen

The semen were from specimens ($N = 25$ cases) remaining after routine andrological analyses. Each specimen had negligible round cells. The study had previously been approved by the Institutional Review Board. Parameters predictive of male fertility were examined including concentration, rapid progression, strict normal morphology, heat-induced hyperactivation (10) and the capacitation index from the sperm

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penetration assay. The capacitation index has been correlated to sperm fertilizing capacity (11). A portion of each specimen was washed by two-layer colloid procedure (12) whereas the remaining portion was frozen (freezing data not shown) for up to 6 months. The semen were thawed (37°C, 10 min) and tested for cell-free DNA.

Modified Isocratic Capillary Electrophoresis (MICE)

The system described here was similar to the horizontal agarose gel electrophoresis with the difference being that the agarose gel was inside a capillary tube to enhance detection of trace amounts of cell-free DNA (9). Each aliquot of semen (50 μL) was drawn into a glass micro-hematocrit capillary tube (internal diameter 1.2 mm, wall 0.2 mm, VWR Scientific Inc., San Francisco, CA). One end of the capillary tube was used to pierce a 2-cm thick block of 2.4 % agarose gel forming an agarose plug. Each capillary tube was sealed at the other end with 10 μL of Liquasonic conducting gel (Chester Labs, Inc., Cincinnati, Ohio). It was important to ensure that there were no air bubbles trapped inside each capillary tube which might interfere with the migration pattern of nucleic acids. The tubes were submerged in 1 × TAE (0.04 M Tris-acetate with 0.001 M EDTA) buffer and a constant voltage of 100 volts was applied for 24 min.

After electrophoresis, each agarose plug was expelled on to a glass slide, held in position with a drop of fast-drying glue applied to the plug end exposed to the TAE buffer. The glass slide with the attached agarose plug was submerged and stained with SYBR Gold fluorescent stain (Molecular Probes Inc., Eugene, OR), rinsed and examined using UV epi-fluorescent microscope at 40 X magnification. Fluorescent images were recorded by placing a digital camera to one of the microscope eyepieces and recording the 640 × 480 pixels images to hard-disk for pixel-intensity analyses. A modification for the digital camera included placing a 4-mm foam ring or large latex washer over the eyepiece to obtain the correct focal point. A 1 Kb DNA ladder marker was included in each experiment to track the cell-free DNA fragment sizes.

Statistical Analysis

To control for interassay variation and background, the data were expressed as an index or ratio of the DNA intensity to the intensity of a control specimen.

Table I. Modified Isocratic Capillary Electrophoresis Detection of Cell-free DNA in Semen ($N = 25$ cases) and the Correlation with Sperm Parameters

Parameter	Correlation coefficients with relative intensities of of DNA fragments	
	High molecular weight (12 Kb)	Low molecular weight (1 Kb)
Sperm concentration (million/mL)	−0.06	0.24
Rapid progression (%)	0.15	0.32*
Normal strict morphology (%)	0.14	0.51*
Capacitation index	0.0	0.34*
Curvilinear velocity	0.27	0.35*
Post-wash hyperactivation (%)	0.27	−0.48*
Heat-induced hyperactivation (%)	−0.42*	−0.32
Abstinence period (days)	−0.13	0.37

* Significant correlation, $P < 0.05$.

The mean pixel intensities were measured at four random points at the agarose tip (high-molecular weight fragments; 12 Kb) and at another four separate points at 4 mm from the agarose tip (low-molecular weight fragments; 1Kb). Data were tested using linear regression and the Student's t-test statistics.

RESULTS

Cell-free DNA was detected in all semen specimens. The quantity of low-molecular weight cell-free DNA was directly related to rapid progression, curvilinear velocity, normal strict morphology and the capacitation index (Table I). Faster swimming sperm (curvilinear velocity >40 μ/s) were associated with more cell-free DNA (Table II). In contrast, post-wash hyperactive motility and heat-induced hyperactivation were negatively correlated to low- and high-molecular weight cell-free DNA respectively. Sperm concentration, linearity, head dimensions, semen volume, pH and postwash total motility were not related to cell-free DNA. A study of fresh versus frozen semen in split specimens did not indicate a difference in detected high-molecular weight cell-free DNA (fresh index 0.67 ± 0.03 mean pixels ± SEM versus frozen index 0.81 ± 0.19, $P > 0.05$). A reduction in the low-molecular weight cell-free DNA after freezing was observed (fresh index 1.50 ± 0.47 versus frozen index 0.52 ± 0.09, $P < 0.05$).

DISCUSSION

The most important finding was the presence of cell-free DNA in ejaculated semen. Present dogma argued

Table II. Modified Isocratic Capillary Electrophoresis Detection of Cell-free DNA in Semen ($N = 25$ cases) and the Correlation with Sperm Parameters. Values are Shown as Mean ± SEM

Parameter	Category	N	Relative intensities of DNA fragments: High molecular weight (12 kb)	Relative intensities of DNA fragments: Low molecular weight (1 kb)
Sperm concentration	≤20 million/mL	10	0.97 ± 0.20	0.65 ± 0.11
	>20 million/mL	14	0.86 ± 0.14	0.59 ± 0.13
Rapid progression	≤25%	5	0.77 ± 0.31	0.47 ± 0.18
	>25%	19	0.94 ± 0.12	0.65 ± 0.10
Normal strict morphology	≤4%	12	0.76 ± 0.17	1.06 ± 0.15
	>4%	12	0.49 ± 0.10	0.73 ± 0.13
Sperm capacitation index	≤7	17	0.87 ± 0.14	0.53 ± 0.08
	>7	7	0.99 ± 0.22	0.81 ± 0.21
Curvilinear velocity	≤40μ/s	9	0.79 ± 0.19	0.43 ± 0.13
	>40 μ/s	15	0.97 ± 0.14	0.72 ± 0.11*
Postwash hyperactivation	≤3%	14	0.80 ± 1.13	0.83 ± 0.11*
	>3%	9	0.96 ± 0.22	0.32 ± 0.06
Heat-induced hyperactivity	≤3%	11	1.07 ± 0.16	0.79 ± 0.14*
	>3%	12	0.67 ± 0.15	0.48 ± 0.10

*Different within same parameter $P < 0.05$.

against the presence of cell-free DNA in semen emphasizing the role of ubiquitous exonucleases that destroyed all nucleic acids. In this study, only the seminal plasma cell-free DNA of low-molecular weight (about 1 Kb) were related to specific sperm parameters such as fertilizing capacity (10,11), in contrast to the high-molecular weight cell-free DNA data. This suggests the possible use of low-molecular weight cell-free DNA as a marker of semen quality. The freezing process reduced the intensity of the low-molecular cell-free DNA possibly due to further fragmentation. To overcome this, the intensity measurements were expressed as an index relative to the control. Since the strict normal sperm morphology has been linked to sperm fertility, the morphology data here suggested that an increase in low-molecular weight cell-free DNA would be associated with sperm function. Interestingly, an increase in low-molecular weight cell-free DNA has been suggested as a cancer marker (5,13,14). Spermatogenesis and cancer are related through rapid cell growth and division. Identifying markers of defective spermatogenesis such as cell-free DNA will aid in making a diagnosis of male reproductive problems.

The source of cell-free DNA was not sperm lysis through freeze-thaw as shown by the results. Note that sperm cells are different from other cell types in that the sperm DNA is tightly condensed and held by disulfide bonds and are not easily lysed by standard cell lysis buffers. Furthermore, the data were analyzed relative to control specimens to reduce confounding variables. In addition, genitourinary tract infected specimens were not included in this study. It is suggested that the source of cell-free DNA in semen might be cells undergoing spermatogenesis (e.g. secondary spermatocytes and spermatids) in close proximity to the seminiferous tubule lumen, accessory sex organ cells (e.g. prostate cells) or reproductive tract cells. Other researchers have reported cell-free DNA resulting from either apoptosis and lysis or active secretion resulting from gene over-amplification or aborted mitosis (5,13,15). Overall, the studies seem to lean toward active secretion of extraneous DNA. The results suggest that the presence of cell-free DNA, regardless of the source, might serve as a marker of fertility-related problems in the male reproductive system.

The function of male cell-free DNA deposited in the female remains unknown. Fetal DNA leaked into the maternal circulation is a marker for gender, intrauterine growth, rhesus D, gene disorders or microchimerism (1,2). Unlike fetal DNA, male DNA in the female circulation may function in horizontal transfers for DNA repair and prime anti-nuclear autoantibodies against viral DNA thus imparting a possible beneficial effect (16,17). Nevertheless, the fate of male cell-free DNA in the female system is interesting and deserves further attention.

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