ANDROLOGY

Tenacity of Exogenous Human Papillomavirus DNA in Sperm Washing

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Purpose: Sperm cells have been shown to take up exogenous DNA readily. The hypothesis was that sperm washing would remove exogenous viral DNA infecting sperm cells. The objective was to compare three types of sperm washing procedures for their capacity to remove exogenous human papillomavirus (HPV) DNA from infected sperm.

Methods: Prewashed sperm were equally divided and sperm in one portion were exposed to L1 HPV DNA fragments for 30 min at 37°C. Untreated washed sperm served as the control. After transfection, the sperm were washed by either centrifuge, two-layer Isolate colloid wash, or test-yolk buffer procedures. Sperm parameters were measured on a Hamilton Thorn HTM-C analyzer. Sperm DNA were extracted and polymerase chain reaction (PCR) was carried out targeting the L1 consensus gene of HPV and the designated sentinel gene, 17q21 spanning the D17S855 gene. Amplified products were analyzed in 2% agarose gel electrophoresis.

Results: PCR analyses detected the consensus L1 HPV gene in sperm after they were processed through either of the three procedures. Controls were negative for the L1 gene. Extracted DNA were verified by PCR amplification of 17q21 spanning the D17S855 gene. Transfected sperm had higher percentages of total motility and progression compared with the control. Centrifuged, washed, transfected sperm exhibited a greater curvilinear velocity and hyperactivation.

Conclusions: The data showed that washing would not remove exogenous HPV DNA from sperm cells. The viral DNA was tenaciously bound to the sperm, suggesting an

internalization into the sperm. The viral DNA also increased the motility of the sperm by affecting the velocity and progression of the sperm, which suggested either an increase in metabolism, an enhancement of the calcium-regulated motility mechanism, or an artifact of PCR reagents. More studies are needed to elucidate the mechanism of DNA stimulated sperm motility.

KEY WORDS: human papillomavirus; sperm washing; exogenous DNA; foreign DNA; Isolate wash.

INTRODUCTION

Human papillomavirus (HPV), particularly types 16, 18, 6, and 11, has been shown to reside in male genital areas (1), in urine, and in semen (2). The uptake of HPV DNA by sperm cells has been reported (1–3) and reviewed (4). In the sperm, the exogenous DNA absorption process is rapid, requiring only 15 min (5). Studies showing that this process is reversible and that the exogenous DNA can be removed by washing is lacking. It is important to patients undergoing assisted reproductive technologies if it can be shown that a specific sperm washing procedure can remove undesirable contaminating viral DNA from sperm. Hence, the hypothesis of the present study was that sperm washing would remove exogenous viral DNA infecting sperm cells. The objective was to compare the capacity of three types of sperm washing procedures in the removal of HPV DNA from infected sperm.

MATERIALS AND METHODS

Preparation and Infection of Sperm Cells

Semen specimens from a known HPV-negative donor were prewashed using the discontinuous two-

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layer 90:47% colloid (Isolate; Irvine Scientific, Santa Ana, CA) gradient centrifugation wash (6). The final sperm pellets were resuspended in modified human tubal fluid (HEPES-HTF; Irvine Scientific, Santa Ana, CA) supplemented with 0.5 mg/ml human albumin fraction V (Irvine Scientific) at a concentration of 20 million/ml.

The washed sperm cells were divided equally into two portions. The treatment consisted of exposing the sperm cells to L1 HPV DNA fragments for 30 min at 37°C. These fragments were previously amplified by polymerase chain reaction (PCR) targeting the consensus L1 gene in HPV-positive cervical condyloma tissues. The remaining portion of untreated washed sperm served as the control.

After 30 min of HPV exposure, the sperm cells were divided and washed either by centrifuge spin-down, by two-layer isolate colloid, wash or by test-yolk buffer wash procedures. The centrifuge wash procedure consisted of mixing an equal volume of modified HTF with the treated sperm, followed by centrifugation at 300g for 10 min. Each sperm pellet was resuspended in 0.4 ml of modified HTF. An aliquot (10 μ l) was removed and sperm parameters were analyzed using the Hamilton Thorn HTM-C analyzer as described below. The remaining sperm cells were pipetted into sterile microfuge tubes and centrifuged at 2700g for 10 sec, the supernatant decanted, and the sperm pellet frozen until used.

The two-layer Isolate colloid wash (6) consisted of layering the HPV-treated sperm on top of a two-layer 90:47% discontinuous colloid gradient inside a 15-ml centrifuge tube. Each colloid gradient was prepared by pipetting 1.0 ml of the 90% colloid solution to the bottom of the 15-ml centrifuge tube and gently layering the 47% colloid solution on top of the 90% layer. The tubes were centrifuged for 20 min at 300g. The supernatants were decanted, and each pellet was resuspended in 1 ml of modified HTF and recentrifuged for 10 min at 300g. The pellet was resuspended in 0.4 ml of modified HTF and an aliquot was removed for analysis. The remaining sperm cells were spun down as described above and the pellet frozen until used.

The test-yolk buffer (TYB) wash consisted of mixing an equal volume of test-yolk medium (Refrigeration Medium; Irvine Scientific) and the sperm suspension, followed by refrigeration (4°C) for 1 hr. After refrigeration, the sperm cells were heat-shocked at 37°C for 5 min, followed by centrifugation at 300g for 10 min. The resultant pellet was resuspended in 0.4 ml of modified HTF and an aliquot analyzed as

detailed below. The remaining sperm pellet was frozen until used.

Computer-Aided Sperm Analyses

Sperm kinematic parameters were measured using the Hamilton Thorn motility analyzer (HTM-C) CASA system as described previously (7). Briefly, the analyses were carried out using aliquots (10 μ l each) of the processed sperm in 20- μ m-deep glass slides with coverslips prewarmed at 37°C on a slide warmer. A minimum of 100 sperm cells was analyzed each time. The percentage of hyperactive sperm was measured using the sort fraction module of the HTM-C based on published settings on this analyzer (7). For consistency, the same observer was used throughout the study.

The parameters measured on the HTM-C were the mean amplitude of lateral head displacement (ALH; distance the sperm head swings from side to side), total motility (percentage of motile sperm which fits the computer criterion for a motile cell), beat cross frequency (BCF; crossing of sperm over the computer-calculated mean path), curvilinear velocity (VCL; velocity calculated from the sum of trackpoint-to-trackpoint velocity), average path velocity (VAP; velocity along a computer curvilinear mean), straight-line velocity (VSL; velocity calculated from the beginning to the end of a digitized sampling interval), and linearity (calculated from the ratio of VSL/VCL).

PCR Analyses of L1 HPV DNA and Sentinel DNA (17q21 Spanning D17S855)

The frozen sperm pellets were thawed and 200 μ l of MilliQ-UF purified water and 50 μ l mineral oil were added to each tube. The sperm cells were boiled at 100°C for 1 hr to release the DNA. An aliquot (10 μ l) of each DNA solution was added to the PCR reaction cocktail (Perkin Elmer Cetus, Norwalk, CT) in a reaction tube (8). Each reaction tube of cocktail reagents contained 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 4.0 mM MgCl₂, a 200 μ M concentration of each dNTP, 100 μ g/ml gelatin, a 1 μ M concentration of each pair of primers targeting the L1 consensus gene of HPV, 1.3 U Taq polymerase enzyme (AmpliTaq; Perkin Elmer Cetus), and 50 μ l mineral oil. The PCR amplified a 450-base pair (bp) product for the L1 gene common for 25 types of HPV. The negative control consisted of the PCR cocktail reagents and purified water. The PCR was carried out in a Perkin Elmer Cetus thermal cycler. A hot-start denaturation

step involving heating each batch of DNA for 7 minutes at 95°C prior to PCR being carried out. The cycling parameters were set for 40 step-cycles with 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C, followed by a final extension at 72°C for 7 min. Each amplified product was pipetted out, mixed with 5× loading buffer, and loaded into the wells along with markers, and 2% agarose gel electrophoresis was performed (8).

The PCR reactions to verify the presence of DNA in each tube targeted the designated sentinel gene of 17q21 spanning the D17S855 gene, which was chosen based on available primers at this laboratory. The cocktail reagents in each reaction tube were the same as mentioned above. The PCR amplified a 434-bp product. PCR was carried out in a Perkin Elmer Cetus thermal cycler. The hot-start denaturation step was carried out as described above. The cycling parameters were set for 32 step-cycles, with 40 sec of denaturation at 94°C, 40 sec of annealing at 60°C, and 40 sec of extension at 72°C, followed by a final extension at 72°C for 7 min. Each amplified product was pipetted out, mixed with 5X loading buffer, and loaded into wells along with markers, and 2% agarose gel electrophoresis was performed.

Data Analyses

The sperm kinematic measurements are presented as the mean $\pm$ standard error (SE) of the mean. Means were analyzed by paired *t* test; *P* < 0.05 was considered significant.

RESULTS

The PCR screening test detected the consensus L1 HPV gene in sperm after they were processed through one of three types of wash procedures: the centrifuge wash, the isolate colloid wash, or the TYB wash (Fig. 1). Washed sperm that were not exposed to the exogenous HPV DNA were negative for the L1 gene. The L1 gene was not detected in the negative controls. The presence of extracted DNA in the sample tubes was verified by PCR of the designated sentinel gene, 17q21 spanning the D17S855 gene (Fig. 2).

Washed sperm cells transfected with the exogenous HPV DNA had higher percentages of total motility and progression compared with the untreated control (Table I). HPV DNA transfected sperm washed by the centrifuge wash procedure, on the average, moved with a higher velocity, particularly curvilinear velocity. Furthermore, these sperm displayed the highest hyperac-

Fig. 1. Failure of different sperm washing procedures to remove exogenous human papillomavirus consensus L1 DNA in sperm cells. Agarose gel electrophoresis (2% gel) of amplified products was carried out after polymerase chain reaction. Experiments were carried out in duplicate. Lane 1, positive control consisting of donor sperm transfected with L1 DNA; Lanes 2 and 3, centrifuge-washed sperm; Lanes 4 and 5, test-yolk buffer-washed sperm; Lanes 6 and 7, two-layer isolate colloid-washed sperm; Lane 8, untreated washed sperm; Lanes 9 and 10, unwashed semen specimen; Lane 10, negative control consisting of water and reagents. Lane M is the *HaeIII* digest of a ϕ X175 marker.

tive motility, in contrast to transfected sperm washed by the Isolate colloid procedure, which showed the lowest hyperactivation. There were no significant differences in the remaining sperm kinematic parameters.

DISCUSSION

The results of this study demonstrated that the three sperm washing procedures failed to remove contaminating HPV DNA from sperm (Fig. 1). The detection of HPV gene sequences in sperm after processing the sperm through the different wash procedures suggested the tenacity of the HPV DNA in sperm. Several reports (4,5,9) have confirmed that sperm cells have the capacity to take up exogenous DNA but there are no data to show if the exogenous DNA can be washed away. Using the three wash procedures followed by PCR analyses, it was shown here that sperm cells could not be washed clear of contaminating HPV DNA. Whether the HPV DNA was incorporated in the genome or mitochondrial DNA was beyond the scope of this study.

Fig. 2. DNA check: verifying the presence of DNA in sperm washed by different procedures using polymerase chain reaction targeting a sentinel gene in 17q21 spanning D17S855. Lane 1, positive control consisting of L1-transfected donor sperm; Lanes 2 and 3, centrifuge-washed sperm; Lanes 4 and 5, test-yolk buffer-washed sperm; Lanes 6 and 7, two-layer isolate colloid-washed sperm; Lane 8, untreated washed sperm; Lane 9, negative control. Lane M is the *HaeIII* digest of a ϕ X175 marker.

Table I. Sperm Parameters Before and After Different Procedures of Washing Infected Sperm: Prewashed Donor Sperm Were Initially Exposed to Human Papillomavirus Consensus L1 DNA for 30 min Before the Wash Procedures^a

Parameter	Raw semen (N = 523)	Sperm			
		Control, washed (N = 498)	Transfected, centrifuge-washed (N = 114)	Transfected, isolate-washed (N = 102)	Transfected, TYB-washed (N = 103)
Total motility (%)	51.5 ± 0.15	75.0 ± 0.45*	90.0 ± 0	93.0 ± 0.10	94.0 ± 0.40
Progressive (%)	15.5 ± 0.11	19.0 ± 0.04*	38.5 ± 0.61	33.5 ± 0.05	37.0 ± 0.70
Average path velocity (μm/sec)	34.0 ± 0.04	41.0 ± 0	47.5 ± 0.05	37.5 ± 0.25	36.0 ± 0.20
Curvilinear velocity (μm/sec)	43.5 ± 0.02	57.5 ± 0.02	73.5 ± 0.05*	56.5 ± 0.25	58.5 ± 0.05
Straight-line velocity (μm/sec)	23.5 ± 0.02	24.5 ± 0.07	31.5 ± 0.2	26.0 ± 0.10	26.0 ± 0.10
Amplitude of lateral head (μm)	1.9 ± 0	3.1 ± 0	4.1 ± 0.03	3.6 ± 0.12	3.7 ± 0.01
Beat cross frequency (Hz)	7.9 ± 0.20	10.8 ± 0.01	11.0 ± 0.12	9.8 ± 0.04	12.0 ± 0.16
Hyperactivation (%)	0.5 ± 0.02	4.0 ± 0.09	8.0 ± 0.38*	1.0 ± 0.10*	4.0 ± 0
Linearity (%)	57.0 ± 0.04	44.5 ± 0.11	44.5 ± 0.14	47.0 ± 0	46.5 ± 0.35

^a Data presented as mean ± SE.* $P < 0.05$.

and has been addressed elsewhere (10). However, what is known is that during fertilization, the entire sperm head and accompanying exogenous DNA are engulfed by an endocytosis-like process into the oocyte facilitated by microvilli projections from the oolemma (4). The uptake of foreign DNA during fertilization is an appropriate concern, particularly in the in vitro environment typified in assisted reproduction technologies.

An unexpected finding was the enhanced motility in sperm transfected with the L1 gene of HPV DNA. This may be an artifact due to the presence of contaminating PCR reagents such as magnesium chloride. However, the possibility exists that exogenous DNA entering the sperm triggers activities related to increased motility. In particular, it was noted that progressive motility was increased, and in the case of centrifuge-washed sperm, the VCL and hyperactivation parameters were significantly higher than those of the controls. These two parameters have been shown to be strongly associated with the fertilizing capacity of the sperm (7,11). A recent report showed that HPV infection of sperm cells resulted in decreased motility (12). This was based on ongoing HPV infection detected in patients and was different from the intentional transfection of sperm with naked HPV DNA molecules in the present study. Clearly, more studies are needed to resolve the mechanisms involved in exogenous DNA stimulated motility in the sperm.

In summary, the study demonstrated that exogenous L1 HPV DNA taken up by sperm cells could not be removed by wash procedures. The exogenous DNA also stimulated sperm motility and progression, and the mechanism involved in this event will require further studies.

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