Human papillomavirus (HPV) infection has been demonstrated in the sperm of a large percentage of sexually active males and is associated with an impairment of sperm parameters, with a particular negative impact on sperm motility, suggesting a possible role in male infertility. Conventional sperm selection techniques have a low efficiency in removing HPV.

**METHODS:** Evaluation of sperm parameters, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling test to evaluate DNA fragmentation and fluorescence *in situ* hybridization or immunohistochemistry for HPV were performed on semen samples from infected patients (n = 22), control subjects (n = 13) and on pooled control sperm samples incubated with HPV16-L1 (HPV capsid), before and after direct swim-up and modified swim-up (with added Heparinase-III). Moreover, cytofluorimetry for HPV detection was performed in pooled sperm pre- and post-incubation with HPV 16-L1 before and after direct and modified swim-up. Statistical analysis was performed with a two-tailed Student’s t-test.

**RESULTS:** Direct swim-up reduces the number of HPV-infected sperm by ~24% (P < 0.01), while modified swim-up is able to remove completely HPV DNA both from naturally and artificially infected sperm. Enzymatic treatment with Heparinase-III tended to decrease sperm motility, viability and DNA integrity but the effects were not significant.

**CONCLUSIONS:** This study shows that Heparinase-III treatment seems not to affect spermatozoa *in vitro* and suggests that this treatment should be investigated further as a means of preparing sperm from patients who are infected with HPV in order to reduce the risk of HPV infection when using assisted reproduction techniques.

**Key words:** assisted reproduction / human papillomavirus / male infertility / sperm infection / sperm selection
When HPV semen infection was present, a strong association with an impairment of sperm parameters, especially a reduction in sperm motility, was observed. These observations raise important concerns about the possibility that sperm cells act as possible vectors for virus transmission to the uterus, allowing vertical transmission to the embryo and even to infant. The literature shows that HPV DNA can be detected in the amniotic fluid (Rogo and Nyanzerwa 1989; Armbruster-Moraes et al., 1994; Xu et al., 1998), fetal membranes (Wang et al., 1998) and even in placental trophoblastic cells (Favre et al., 1998), syncytiotrophoblastic cells of spontaneously aborted material (Hermonat et al., 1997), cord blood specimens of neonates (Tseng et al., 1992) and oropharyngeal secretion of newborns (Alberico et al., 1996). In these sites, infection might occur either through a vertical transmission such as ascending infection from the cervix or horizontal transmission via infected sperm at conception. At present, the possible consequences of fetal exposure to HPV are not well defined. However, in vitro experiments showed that HPV-transfected trophoblast cells have an increased rate of apoptosis and a reduced placental invasion into the uterine wall, compared with controls (Calinisan et al., 2002; Lee et al., 2002; Henneberg et al., 2006). Moreover, artificially infected sperm, either transfected with E6/E7 HPV genes or incubated with viral proteins, are able to enter the oocyte to deliver HPV genome and viral genes are then actively transcribed by the penetrated oocyte (Foresta et al., 2011a,b,c). Interestingly, a recent study demonstrated that infected couples undergoing assisted reproduction technique (ART) cycles experienced an increased risk of pregnancy loss compared with non-infected couples (Perino et al., 2011). In these couples, the highest predictive factor for early abortion was represented by HPV sperm infection. A significant presence of HPV DNA has also been reported in thawed semen samples from healthy sperm donors (Foresta et al., 2010) and from patients who banked their sperm because of chemo/radiotherapy (Kaspersen et al., 2011). Considering that cryopreserved semen and sperm samples from infertile patients are frequently used in ART (IVF and ICSI), it is of paramount importance to eliminate HPV infection from sperm cells before use. Recent findings have demonstrated a high persistence of HPV in semen samples treated with different sperm selection procedures and that conventional procedures and culture media have a low efficacy in eliminating HPV infection from sperm (Foresta et al., 2011a,b,c).

Some authors have demonstrated the adhesion of HPV DNA to the surface of sperm cells, localized at two distinct binding sites along the equator of the sperm head (Pérez-Andino et al., 2009; Foresta et al., 2010). As previously demonstrated for human immunodeficiency virus (HIV), they showed that the glycosaminoglycans (GAGs) mediate the viral binding to sperm. It has been demonstrated that the GAG syndecan-1 is able to interact with the viral capsid protein L1 on the sperm (Foresta et al., 2011a,b,c).

Many studies have been performed on the management of HIV-infected semen in serodiscordant couples, with affected men requiring ART to achieve a safe conception. With this aim, Ceballos et al. (2009) obtained a significant reduction of GAGs expression on sperm and consequent inhibition of HIV-L1 sperm capture by using a sperm selection method with an enzyme treatment.

We performed the present study to evaluate the effectiveness of a modified swim-up, with added Heparinase-III to remove HPV DNA from naturally and artificially infected semen samples. Our hypothesis is that Heparinase-III treatment will cleave heparin sulfate sperm binding and thereby prevent HPV attachment to sperm.

**Materials and Methods**

**Collection and preparation of semen samples**

Semen samples from 22 HPV-infected patients and from 13 nonmozoospermic non-infected volunteers (control subjects) were obtained by masturbation after 2–5 days of sexual abstinence and stored in sterile containers. The presence or absence of HPV in semen samples of all participants had been previously evaluated by PCR. All participants had provided informed consent and the study was approved by the Institutional Ethics Committee of the Hospital of Padova, Italy, by the Protocol No. 2336. Samples were allowed to liquefy for 30 min and semen volume, pH, sperm concentration, viability, motility and morphology were determined following World Health Organization (2010) guidelines for semen analysis. All samples had normal viscosity, pH and semen volume. The presence of sperm antibodies, varicocele and seminal infections were evaluated, respectively, by Sperm-Mar test (Ortho Diagnostic System, Milan, Italy), testicular Doppler ultrasound and microbiological sperm culture.

A pool of sperm, prepared using an aliquot of semen from each control subject, was artificially infected by incubation with an HPV capsid, HPV16-L1 (L1-incubated) and used for further analysis. The three groups (infected patients, controls and L1-incubated sperm) were then analyzed by the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) test, fluorescence in situ hybridization (FISH) for HPV (infected patients and controls) and immunohistochemistry (IHC) for HPV (incubated sperm and controls) before and after direct swim-up and modified (plus Heparinase-III) swim-up. Pooled sperm was analyzed also by cytofluorimetry analysis for HPV to confirm the artificially acquired infection and was re-evaluated after HPV 16-L1 incubation before and after modified swim-up. Sperm parameters were re-evaluated in the three groups after direct and modified swim-up.

**TUNEL assay**

Sperm cells were centrifuged (600 g for 4 min), washed in Sperm Washing Medium (SWM, Irvine Scientific, CA, USA) and fixed with 2% paraformaldehyde for 15 min on ice. After fixation, the spermatozoa were centrifuged (600 g for 4 min) and washed with phosphate-buffered saline (PBS). Spermatozoa were then resuspended in 100 μl of fresh premeabilization solution (10 mg sodium citrate, 10 μl Triton X-100 in 10 μl H2O) and incubated for 2 min at 4°C. Cells were then centrifuged (600 g for 4 min), the pellets washed with PBS and then incubated in 2% EDTA in PBS for a period of 45 min at room temperature. Cells were then centrifuged again (600 g for 4 min) and the pellets washed with PBS. The positive control samples were treated with 100 μl of DNase I (Roche Diagnostics, Indianapolis, IN, USA); 1 mg/ml supplemented with 10 μl MgSO4 (100 mm) for 1 h at room temperature. TUNEL analysis was performed with the In Situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer’s instructions. Spermatozoa were then washed twice in PBS, diluted to a final volume of 300 μl in PBS and kept in the dark for analysis via flow cytometry.

**FISH for HPV in human sperm**

Sperm samples were treated as previously described (Foresta et al., 2011a,b,c). Briefly, samples were incubated with 20 μl of hybridization solution containing biotin-labeled HPV DNA probe (Rembrandt in situ hybridization and detection kit HPV, Pan Path, Amsterdam, Netherlands). After denaturation of cellular target DNA and HPV DNA probe on a heating block for 5 min at 95°C, hybridization was performed by
incubating the samples at 37°C overnight in a humidified chamber. The negative control was processed in the same way but omitting the viral probe. The biotin-labeled HPV probe was detected by incubation with 1:200 streptavidin Texas red (Vector Laboratories, Burlingame, CA, USA) for 40 min at room temperature. After detection, the slides were washed twice in PBS/0.01% Triton and then twice in PBS and mounted with a solution containing 4.6-diamino-2-phenylindole (DAPI, 5 mg/ml). For each sample three slides were prepared and examined. Samples were analyzed using a fluorescence microscope (Nikon, Eclipse E600, Melville, NY, USA) equipped with a triple band-pass filter set (fluorescein isothiocyanate, tetrarhodamine isothiocyanate, DAPI).

### Pooled sperm incubation and detection of HPV16-L1

A pool of sperm from normozoospermic non-infected samples was incubated with HPV16-L1. Pooled sperm was washed twice in SWM, in order to eliminate seminal plasma. Samples were divided into two aliquots and incubated for 1 h at 37°C in fresh SWM with or without HPV16-L1 (1 μg/ml: 1 × 10^6 spermatozoa; Gardasil, Sanofi Pasteur MSD, Lyon, France). This condition proved to be optimal after a series of experiments performed with different times and HPV-L1 concentrations (data not shown). For the detection of HPV16-L1 capsid protein in incubated pooled sperm, we used immunofluorescence as follows: 10 μl of sperm sample was smeared on clean, grease-free slides, air-dried and fixed in PBS/paraformaldehyde 4% for 15 min. The slides were washed three times in PBS for 5 min at room temperature and then used for HPV capsid (HPV16-L1) detection. Sperm were incubated with mouse monoclonal antibody HPV 16-L1 (CAMVIR-I; 0.8 μg/ml, 1:250, Santacruz, Santa Cruz, CA, USA) for 120 min at room temperature, and then washed in 0.2% Tween-PBS for 5 min. Immunoreaction was detected by sequential incubation with biotinylated goat anti-mouse immunoglobulin secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA) and streptavidin Texas red (1:400, Vector Laboratories) both for 60 min at room temperature. The slides were then washed twice in PBS. Nuclei were counterstained with DAPI (5 mg/ml), slides were mounted with anti-fade buffer and 24 × 24 mm coverslip. Immunostaining was evaluated with a Nikon ViCo Video Confocal Microscope.

### Direct swim-up

Direct swim-up was performed placing 1.0 ml of semen sample in a sterile 15-ml conical centrifuge tube and centrifuged at 300 g for 10 min. The supernatant was carefully eliminated and the sperm pellet was resuspended in 0.3 ml of SWM by gentle pipetting. Gently, 1.0 ml of SWM was layered over samples; the tube was inclined at an angle of ~45°, to increase the surface area of the semen–culture medium interface, and incubated for 1 h at 37°C in an incubator with 5% CO₂. The tube was returned to the upright position and the uppermost 0.8 ml of the upper layer was collected, containing the sperm that had swum up. Finally, the collected spermatozoa were used in subsequent analysis.

### Modified swim-up with enzymatic treatment (Heparinase-III)

The direct swim-up technique was modified as follows: after centrifugation and resuspension of sperm pellet, 0.3 ml of sperm washing medium with Heparinase-III was added (2.5 U/ml). This condition proved to be optimal after a series of experiments using different enzyme concentrations (data not shown). Gently, 1.0 ml of SWM with added Heparinase-III was layered over samples; remaining procedures were performed as described above for direct swim-up.

### Statistical analysis

The values shown are the averages of at least three evaluations performed by different operators.

### Table I  Sperm parameters, TUNEL test and detection of HPV-positive sperm in infected patients, control subjects and in sperm incubated with HPV 16-L1 (L1-infected).

<table>
<thead>
<tr>
<th></th>
<th>Semen volume (ml)</th>
<th>pH</th>
<th>Sperm conc. (mill./ml)</th>
<th>Total sperm count (mill.)</th>
<th>Progressive motility (%)</th>
<th>Normal morphology (%)</th>
<th>Viability (%)</th>
<th>TUNEL (%)</th>
<th>FISH/ IHC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-infected patients</td>
<td>3.1 ± 0.9</td>
<td>7.6 ± 0.2</td>
<td>29.0 ± 10.3</td>
<td>87.7 ± 36.3</td>
<td>29.6 ± 14.2</td>
<td>19.0 ± 6.3</td>
<td>81.3 ± 6.3</td>
<td>84.5 ± 4.7</td>
<td>18.6 ± 6.2</td>
</tr>
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<td>(n = 22)</td>
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<td></td>
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<tr>
<td>Control subjects</td>
<td>3.3 ± 1.0</td>
<td>7.5 ± 0.3</td>
<td>30.5 ± 9.8</td>
<td>98.8 ± 46.7</td>
<td>42.4 ± 22.7</td>
<td>21.1 ± 7.5</td>
<td>83.8 ± 8.3</td>
<td>86.3 ± 3.4</td>
<td>0.0/0.0</td>
</tr>
<tr>
<td>(n = 13)</td>
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<tr>
<td>L1-incubated sperm</td>
<td>22.6 ± 8.7**</td>
<td>20.9 ± 6.5</td>
<td>82.8 ± 8.7</td>
<td>85.9 ± 5.0</td>
<td>82.4 ± 7.3</td>
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<tr>
<td>(pool)*</td>
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<td></td>
</tr>
</tbody>
</table>

L1-incubated, sperm incubated with HPV 16-L1; FISH, fluorescence in situ hybridization, IHC, immunohistochemistry, TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (% TUNEL' refers to the percentage of sperm in the sample which showed DNA fragmentation).

*Sememen volume, pH, sperm concentration and total sperm count are not reported because of the pooling process.

*P < 0.05 versus control subjects (Student’s t-test). Data are mean ± SD.

**P < 0.01 versus control subjects.
Results

Patients with HPV semen infection had a mean age of $36.7 \pm 3.8$ years no different from normozoospermic subjects. Antisperm antibodies, varicocele and seminal infections were excluded in all samples. Table I reports the mean sperm parameters, FISH and/or IHC analysis for HPV-infected patients and on normozoospermic subjects before (controls) and after pooling and incubation with HPV16-L1 (L1 incubated sperm). Seminal volume, pH, sperm count, normal morphology and viability were not different in HPV-infected and in non-infected semen samples. A reduction of mean progressive sperm motility was found in semen samples of infected patients ($29.6 \pm 14.2\%$, $P < 0.05$) versus controls ($42.4 \pm 22.7\%$) and this reduction was even stronger in pooled sperm incubated with HPV 16-L1 ($22.6 \pm 8.7\%$, $P < 0.01$). The TUNEL test, performed to evaluate sperm DNA fragmentation, showed no difference between infected patients, controls and pooled sperm after incubation with HPV16-L1. FISH analysis for HPV showed a positive result in all samples of infected patients with a mean percentage of positive sperm of $18.6 \pm 6.2\%$ (ranging from 6 to 31%). Semen samples from control subjects served as a negative control for the same analysis. IHC for L1, used to verify the presence of HPV capsid protein on incubated sperm, showed a positive result of $82.4 \pm 7.3\%$ (ranging from 67 to 93%), while controls were negative.

Comparing samples obtained by direct swim-up and by modified swim-up (with addition of Heparinase-III), we observed no differences in terms of sperm motility, viability and DNA fragmentation (Table II). Although a decreasing trend was present in samples treated with Heparinase-III, the sperm viability and DNA fragmentation were better than for pretreatment. Table III reports the mean percentage of HPV-infected sperm detected by FISH and/or IHC in the three groups before and after direct and modified swim-up. While controls were always negative for both FISH and IHC, infected patients and L1-incubated sperm were positive, both pre-and post-direct swim-up. After Heparinase-III treatment, both samples from infected patients and the artificially infected pool were negative in FISH and IHC. Fig. 1 shows the flow cytometry analysis performed in pooled sperm before and after incubation with HPV 16-L1 and post-Heparinase-III treatment. The logarithmic plot clearly illustrates the different distribution of sperm cells from controls and incubated samples. Interestingly, sperm cells incubated with L1 (black line), after treatment with Heparinase-III (grey line), were distributed similarly to that of controls (dotted line). Fig. 2 shows pictures obtained by FISH and IHC analyses performed on samples of infected patients, control subjects and L1-incubated sperm before and after direct and modified swim-up. The presence of the virus and L1 protein is evident in samples from infected and L1-incubated semen. After swim-up the samples showed a reduced fluorescence ($18.6 \pm 6.2\%$ basal versus $4.5 \pm 1.5\%$ post-swim-up, $P < 0.01$ and $82.4 \pm 7.3\%$ basal versus $19.8 \pm 6.8\%$ post-swim-up, $P < 0.01$, respectively).

Data are presented as mean $\pm$ SD. Differences between data were determined by two-tailed.

Student’s t-test after confirmation of normal distribution with the Kolmogorov–Smirnov test. P-values (two sided) $<0.05$ were considered statistically significant.

<table>
<thead>
<tr>
<th>Table II</th>
<th>Sperm parameters and TUNEL test results in infected patients, control subjects and in sperm incubated with HPV 16-L1, before (basal) and after direct and modified swim-up.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viability (%)</td>
</tr>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>HPV-infected patients ($n = 22$)</td>
<td>81.3 $\pm$ 6.3</td>
</tr>
<tr>
<td>Control subjects ($n = 13$)</td>
<td>83.8 $\pm$ 8.3</td>
</tr>
<tr>
<td>L1-incubated sperm (pool)</td>
<td>82.8 $\pm$ 8.7</td>
</tr>
<tr>
<td>No significant differences were observed. Data are mean $\pm$ SD.</td>
<td></td>
</tr>
</tbody>
</table>
while they were all negative after Heparinase-III treatment. Control samples were always negative.

**Discussion**

Sexually transmitted diseases may affect the health and fertility of both men and women. HPV is the agent of most common sexually transmittable infection and in some cases can lead to warts and cancers (Graziottin and Serafini, 2009; Stanley, 2010; Nyitray et al., 2011). A high incidence of HPV infection has been demonstrated in sperm from sexually active men, even if asymptomatic, and recently the hypothesis of HPV as a novel cause of male infertility has been suggested (Rintala et al., 2004; Foresta et al., 2010; Foresta et al., 2011a,b,c). Hermonat et al. (1997) reported that HPV infection was more prevalent in spontaneous abortion specimens compared with specimens from elective abortions, strengthening the hypothesis of a major role of this virus in infertility, and in particular in miscarriages. Recently, a study investigating the role of HPV infection in infertile couples undergoing ART showed a correlation between HPV male infection and increased risk of pregnancy loss after ART (Perino et al., 2011). In accordance with these findings demonstrating the tenacity of HPV DNA binding to sperm, in a previous study using three classic sperm selection techniques (sample washing, discontinuous density gradient and direct swim-up), we observed a reduction of HPV-infected sperm cells but only in a few cases treated by swim-up were we able to eliminate HPV infection (Foresta et al., 2011a,b,c). In light of these observations, it seems of paramount importance to identify a new protocol of sperm selection which can remove HPV infection from sperm cells before their use by ART. In HIV serodiscordant couples requiring ART, an enzymatic treatment for the complete removal of sperm infection has been successfully proposed in order to achieve safe conception (Ceballos et al., 2009). In the present study, we tested a modified swim-up, with an addition of Heparinase-III, to attempt elimination of HPV from infected sperm samples. The rationale of this treatment was to cleave the binding between HPV and its putative ligand, syndecan-I, on the sperm surface. Here we confirmed that naturally infected sperm show a significant reduction of their progressive motility, and the same finding was demonstrated in artificially infected semen obtained by incubation of sperm with the viral capsid protein. This finding supports the hypothesis of the role of HPV as a risk factor for male infertility, even if other sperm parameters and DNA integrity of infected sperm were unaffected. Moreover, FISH analysis showed that patients with HPV semen infection have a high prevalence (~20%) of infected sperm. The tenacity of HPV binding to sperm has been demonstrated, and the direct swim-up was not effective in eliminating the infected sperm from the semen. The application of a modified swim-up showed an impairment of sperm viability, motility and DNA fragmentation that could raise concerns about the clinical use of this technique.

**Table III** HPV detection in sperm samples from infected patients, control subjects and sperm incubated with HPV 16-L1, before and after direct and modified swim-up.

<table>
<thead>
<tr>
<th></th>
<th>FISH/IHC (%)</th>
<th></th>
<th>Swim-up + Hep-III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Swim-up</td>
<td></td>
</tr>
<tr>
<td>HPV-infected patients (n = 22)</td>
<td>18.6 ± 6.2</td>
<td>4.5 ± 1.5*</td>
<td>0.0</td>
</tr>
<tr>
<td>Control subjects (n = 13)</td>
<td>0.0/0.0</td>
<td>0.0/0.0</td>
<td>0.0/0.0</td>
</tr>
<tr>
<td>L1-incubated sperm (pool)</td>
<td>82.4 ± 7.3</td>
<td>19.8 ± 6.8*</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Data are mean ± SD. 
*P < 0.01 versus basal samples.

**Figure I** Cytofluorimetric analysis for HPV in pooled sperm before incubation with HPV 16-L1 (L1-incubated) and after incubation, with or without Heparinase III (Hep-III) treatment.
However, these differences between direct and modified swim-up were not statistically significant. The most important finding of this study concerns the percentage of infected sperm observed before and after treatment with Heparinase-III. Although a significant reduction in the percentage of infected sperm was observed after direct swim-up, interestingly modified swim-up was able to completely eliminate HPV DNA from sperm. This is the first study demonstrating the capability of a modified swim-up technique to select non-infected sperm from HPV DNA-positive semen samples. Moreover, we showed that Heparinase-III treatment at these doses does not significantly affect sperm quality and DNA integrity.

In conclusion, we suggest testing the semen of all idiopathic infertile males with risk factors (warts, positive partners and previous infections) for the presence of HPV because infection of sperm with HPV may represent a novel cause of male infertility. In addition, considering that HPV infection has been suggested to have a role in spontaneous abortions, we propose that couples with recurrent miscarriages should be tested for the presence of HPV in the semen and, in cases with a positive result, the semen treated with an appropriate procedure to obtain sperm which are free of the virus. In this study we confirmed the inadequacy of direct swim-up and demonstrated the efficacy of a modified swim-up, with the addition of Heparinase-III. Although larger studies are needed to confirm our findings before considering clinical application of this method, our results support the possibility of reducing the risk of HPV infection during ART.

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Authors’ roles
All authors had full access to the original data, reviewed the data analysis, contributed to data interpretation and to the writing of the report, made final decisions on all parts of the report and approved the final version of the submitted report. A.L., D.P., G.P. and C.F. participated in study design, A.G. and C.F. enrolled patients. A.B. and A.G. undertook statistical analysis. L.D.T. and A.B. made molecular analysis. A.G., D.P. and A.B. contributed to data collection and generation of tables and figures.

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Conflict of interest
None declared.
References


