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HPV in sperm is efficiently removed by washing

A suitable approach for assisted reproduction

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ABSTRACT

Research question: Is it possible to obtain HPV-free sperm cells to be employed in assisted reproduction from clinically HPV-positive men by sperm washing?

Design: Observational study performed on HPV-positive patients. Freshly ejaculated sperm was collected, readily processed by gradient separation followed by swim-up from the washed pellet. The resulting fractions tested for the presence of HPV DNA.

Results: Of the fifteen clinically HPV-positive subjects, 66% were positive in at least one of the seminal fractions. If any postivity was detected, plasma was always HPV-positive suggesting that HPV homes in seminal plasma. No consistent pattern was observed throughout different samples in cell pellet, round cells and non-motile sperm cells fractions. However, upon sperm-wash procedure, the fraction of motile sperm cells was never found HPV positive.

Conclusions: Although sperm washing approaches failed so far, the procedure used herein was never tested for HPV. Indeed, such sperm washing technique can efficiently remove HPV from sperm. However, the present study was conducted on a small population and a larger follow-up study is recommended. HPV screening should be performed in sperm samples and, upon HPV-positivity, sperm-washing should be considered before assisted reproduction techniques.

KEYWORDS

HPV; sperm wash; assisted reproduction; HPV-positive sperm; HPV-testing.

INTRODUCTION

Human Papilloma viruses (HPV) are a heterogenic family of non-enveloped DNA viruses that commonly infect epithelia. Seventy-five % of the human population is estimated to be HPV positive worldwide (Capra et al., 2015; Giuliano et al., 2011; Koutsky, 1997; Lowy et al., 1994).

In addition to testicular cancer (Garolla et al., 2012b), HPV infection has been related to decreased fertility in male individuals (Boeri et al., 2019; Damke et al., 2017; Donà et al., 2018; Foresta et al., 2010b; Garolla et al., 2012a, 2013, 2018; Gizzo et al., 2014; Luttmer et al., 2016; Moghimi et al., 2019; Tangal et al., 2019; Yang et al., 2013), and to a decreased success rate during *in vitro* fertilization (Depuydt et al., n.d.; Garolla et al., 2016; Henneberg et al., 2006; Noventa et al., 2014; Pereira et al., 2015). In fact, among the couples undergoing assisted reproduction procedures, an increased risk of pregnancy loss is seen in presence of HPV infection and in particular when the male partner is infected (Perino et al., 2011). Confirming these findings, one of the predictive factors for abortion was indeed shown to be HPV positivity (Perino et al., 2011).

Although a steadily increasing literature is focused on the impact of HPV on sperm (Depuydt et al., 2019; Didelot-Rousseau et al., 2007; Giuliano et al., 2011), it is still not clear whether HPV is prevalently localized in the seminal plasma or in the cellular fractions (Capra et al., 2015; Cortés-Gutiérrez et al., 2017; Golob et al., 2014). In fact, multiple authors report HPV positivity of the overall sperm (Foresta et al., 2010b; Garolla et al., 2016; Gizzo et al., 2014; Luttmer et al., 2016; Yang et al., 2013), but just few of them confirm HPV to be present inside spermatic cells *in vivo* (Foresta et al., 2010b, 2011a, 2011b). In fact, Foresta and colleagues observed by fluorescence *in situ* hybridization (FISH) the presence of HPV DNA in the equatorial region of the sperm head in 25% of

sperm cells (Foresta et al., 2010a, 2010b). Interestingly, HPV was never found in sperm cells of fertile subjects (Foresta et al., 2010b). Such observations are supported by Pérez-Andino *et al.*, who confirm the presence of HPV in sperm cells' head (Pérez-Andino et al., 2009). However, such observation were performed upon incubation with the virus, and, therefore, are meant as an *in vitro* model. Recently, Capra and colleagues developed a new approach to evaluate HPV localization in the different semen cellular components (Capra et al., 2019). They observed the presence of HPV DNA, belongings to one or multiple HPV genotypes, in different seminal fractions. Uncertainities about the actual localization of HPV in semen notwithstanding, the observation that HPV infection is associated with decreased fertility is universally accepted. This raises concerns for the role played by the virus within the context of assisted reproduction, especially considering that the prevalence of HPV positive semen among sperm donors (Foresta et al., 2010b) and among those who bank their sperm, due to medical procedures (Kaspersen et al., 2011), is high. For this reason and because HPV-positive sperm increases the risk of infection in female individuals, we investigated the prevalent localization of HPV in sperm fractions. Because classical sperm washing procedures were not shown to remove HPV (Brossfield et al., 1999; Foresta et al., 2011b), we also verified whether a well-consolidated washing technique used to remove HIV from sperm (Savasi et al., 2010, 2007; Semprini et al., 1993; Sunderam et al., 2008; Zafer et al., 2016) could also be effective in getting rid of spermatic HPV and routinely employed as clinical practice.

MATERIAL AND METHODS

Fifteen HPV positive individuals were enrolled in the present study (table I). Enrollment criteria included a healthy BMI between 18 and 25 kg/m^2, no other viral cronic infections desease, no fertility-related pathologies or cause of male infertility including varicocele, previus testicular surgery, endocrine disfunction or cancer. Mycoplasma Genotalium, Chlamydia Trachomatis and

Naisseria Gonorrehoeae infections assested by urethral swabs were addistion exclusion criteria. HPV positive individuals were clinically identified by presence of condyloma. DNA isolated from a donor who never had any sexual intercourse was used as negative control. DNA isolated from condylomas was used as positive control. All samples were previously made anonymous in accordance with the requirements of the Italian Personal Data Protection Code (Legislative Decree No. 196/2003) and the general authorisations issued by the Italian Data Protection Authority. Ethics Committee approval was considered unnecessary because, under Italian law, it is only required in the case of prospective clinical trials of medical products for clinical use (Arts. 6 and 9 of Legislative Decree No. 211/2003). All of the patients had given their informed consent, following Helsinki declaration. Semen samples were obtained by masturbation after 3-7 days of sexual abstinence, analysed and then washed. All samples were analyzed by the same biologist and at the same laboratory. Samples were processed at the laboratory within 2 hours of ejaculation according to World Health Organization (WHO) recommendations (World Health Organization, 2010). Motility was classified according to the WHO criteria as follows: a, rapid progressive spermatozoa; b, slow progressive spermatozoa; c, non-progressive spermatozoa; and d, immotile spermatozoa. The sperm concentration was calculated by a Makler chamber. The total sperm count was calculated as: total sperm count ($x10^6$/mL) x ejaculate volume (in mL). The Papanicolaou smear for staining of spermatozoa was adopted for morphological evaluation. Sperm was bacteria-free in all patients, and the leukocyte count was under the WHO threshold (<1 million/ml). Sperm wash consisted in a first step of separation on a 40–80% density gradient (Pureception kit, Sage) and centrifuged for 30 min at 400 g. This resulted in four different fractions: motile spermatozoa, non-sperm cells, immotile spermatozoa and seminal plasma. The supernatant was removed, and the sperm pellet recovered and resuspended in 3 ml of fresh medium (Sperm-washing medium, Sage). After a wash step (400 g for 10 min), 1 ml of medium

was gently layered on the pellet, and the tube was incubated at 37°C for 1 h. After swim-up, a supernatant volume of ~500 µl was recovered. Such precedure was previously described (Savasi et al., 2007), and is graphically shown in figure 1. Total DNA was extracted from all the five fractions resulting from the washing procedure by DNA purification Maxwell® RSC Instrument (Promega) and quantified by the Nanodrop 2000 Instrument (Thermo Scientific). One µg of DNA was analyzed by nested PCR. When the DNA concentration did not allow to use one µg, the maximum amount of DNA was analyzed, never less than 100 ng. PCR was performed employing readyMix RED Taq Polymerase (Sigma) and MY09/MY11 (product size 450 bp) and GP5+/GP6+ (product size 150 bp) primers (MY09 CGTCCMARRGGAWACTGATC, MY11 GCMCAGGGWCATAAYAATGG, GP5+ TTTGTTACTGTGGTAGATACTAC and GP6+ GAAAAATAAACTGTAAATCATATTC) (Abreu et al., 2012; Bertazzoni et al., 2013; Camargo et al., 2011; Matah and Sareen, 2012; Qu et al., 1997). These primers anneal to L1 region of HPV genome and their sequence is degenerated in order not to be specific for any particular HPV, but to rather be broad spectrum and target the different strains of HPV. As control, samples were tested also for the human *28S* (28S-f TTAAGGTAGCCAAATGCCTCG, 28S-r CCTTGGCTGTGGTTTCGC). PCR products were loaded on 1.5% agarose gel and visualized by Chemidoc (Bio-Rad). Along with PCR product, the 100bp plus ladder (Genespin) was loaded, in order to have a size marker during the electrophoretic run. Products from either PCR reactions were cloned into TOPO-TA cloning vector (Invitrogen) in order to determine the limit of detection in our experimental condition (Fenizia et al., 2014; Vaccari et al., 2014). We were able to detect down to five copies per reaction, consistently with how previously standardized (Qu et al., 1997; Rodrigues et al., 2013).

RESULTS

Fifteen HPV positive individuals were enrolled in this study and their sperm was screened for the presence of HPV DNA by nested PCR. The mean age of the patients was 32.3±7.9 years, and all of them had had an adequate BMI.

All the five fractions resulting from the sperm wash procedure described above were analyzed in each donor. Briefly, fractions were identified as: seminal plasma, cell pellet, round cells, non-motile abnormal sperm cells and motile sperm cells (fig. 1).

In figure 2, the results obtained from six representative individuals are displayed. Positive and negative controls are included as well in the figure.

HPV DNA was not found in five out of fifteen samples (33%) (fig. 2f serves as an example). Samples obtained from the other ten donors (66%) resulted to be positive in at least one of the fractions, as exemplified in fig. 2a, b, c, d, and e. No consistent pattern was observed throughout the different samples in the cell pellet, in the round cells and in the non-motile sperm cells fractions. Notably, whereras HPV DNA was never detected in motile sperm cells, i.e. in the fraction used for fertilization procedures (table II) upon the sperm washing procedure used herein, viral DNA was always present in the fraction derived from seminal plasma, indicating that HPV DNA is localized in seminal plasma and not in motile sperm cells.

Considering that the purpose of this work is to detect the smallest amount of HPV DNA, even the faintest bands, evidence of very few copies, were considered (i.e. fig. 2c, lane 1 and 3). As control for DNA quality and presence of potential polymerase inhibitors, PCR was performed on the human gene 28s as reference. Results are shown below nested PCR panels for each corresponding sample, including positive and negative controls (fig. 2a-f). In each tested fraction, we were able to succesfully detect the intended *28s* amplicon.

DISCUSSION

HPV infection is highly prevalent worldwide, but our knowledge of the role of this virus on fertility is still partial at best. Two open controversial questions, in particular, have not yet been unequivocally answered, one regarding the presence of the virus in the spermatozoa and the other one regarding the potential influence of HPV infection on seminal parameters.

Recent meta-analyses have shown that HPV semen infection is a risk factor for male fertility (Foresta et al., 2015; Lyu et al., 2017; Xiong et al., 2018). However, the specific mechanism underlying this association has yet to be elucidated. Boeri's observations in his recent study confirmed the potentially detrimental impact of HPV seminal detection on sperm progressive motility in a group of infertil men (Boeri et al., 2019; Depuydt et al., 2019). However, in this study, authors evaluated the presence of HPV in the overall sperm, not investigating into depth the different sperm fractions. Capra's experiments were performed in different seminal fractions, similarly to our work. They focused more on the identification of the HPV genotypes in the different fractions (Capra et al., 2019), while we kept our analyses as much "pan-HPV" as possible. In fact, our main focus was to obtain HPV-free sperm cells, suitable for clinical purposes. The results of Capra and colleagues confirms our findings.

We analyzed a small group of HPV-infected man to verify whether in this situation HPV can be detected in semen and, if that was the case, which seminal fraction hosts the virus. Initial results indicated that HPV was present in semen the majority of the individuals analyzed. Analyses of the seminal fraction obtained using well known a sperm separation procedure showed that in every HPV-infected semen the virus is present in the seminal plasma fraction. Notably, HPV could be detected in cell pellet, round cell and non-motile sperm cells fraction as well, but was never observed in the motile sperm fraction.

It is still debated in which kind of ejaculated cells HPV can be found, and whether the virus lies on the cell surface or it is located at the intracellular level (Cortés-Gutiérrez et al., 2017; Foresta et al., 2011a; Garolla et al., 2012b, 2012a; Pérez-Andino et al., 2009). Overall, these observations allow us to speculate about which could possibly be the impact of HPV with sperm cells and the advantage for a virus to infect sperm cells, which in their mature stage are transcriptionally inactive (Williams and Smith, 1996). Pérez-Andino suggests that this could be an efficient strategy to bypass female mucosal protection, in order to enhance virus spread and mucosal penetration (Pérez-Andino et al., 2009). On the other hand, the observation that HPV was never detected in the motile sperm fraction, the one which fertilizes an egg, would be finalized at preventing a disastrous fetal infections. Thus, viral infections are transmitted to the unborn child through a maternal infection. This seems to be a necessary condition for the fetus to become infected. If the sperm cell capable of fertilizing an egg had HPV integrated into its DNA, then a child would be born with all the cells infected with HPV.

Sperm washing procedures had been tested in the past, but none of them was able to succesfully and repeatedly remove HPV from the sperm. (Brossfield et al., 1999; Foresta et al., 2011b; Olatunbosun et al., 2001; Rintala and Gre, n.d.). Herein we utilized a sperm washing procedure optimized for HIV infected individuals (Semprini et al., 1993) and routinely employed in clinical practice (Savasi et al., 2007; Semprini et al., 1993; Sunderam et al., 2008; Zafer et al., 2016). This procedure differs from the previous ones (Brossfield et al., 1999; Foresta et al., 2011b; Olatunbosun et al., 2001; Rintala and Gre, n.d.) since it is based on the use of multiple approaches, including stratification on density gradient, sperm wash and swim-up (Savasi et al., 2010, 2007; Semprini et al., 1993; Sunderam et al., 2008; Zafer et al., 2016). Notably, the use of this procedure resulted a spermatic fraction of motile sperm cells that was consistently HPV negative in all the analyzed samples. In order to analyze such samples, we performed a nested PCR, as previously

standardized (Camargo et al., 2011; Coutlée et al., 2005; Matah and Sareen, 2012; Savasi et al., 2010). This technique is able to detect minimal amount of specific HPV DNA (down to four copies in our hands) in the bulk of one µg of DNA per reaction, corresponding to $400*10^3$ cells, roughly. Results confirmed that the motile sperm cell fraction, the one used in fertilization procedures, was always HPV-free.

We did not observe a consistent pattern of HPV-detection in the cell pellet, in the round cells and in the non-motile sperm cells. Besides being present within infected cells, HPV can be found attached to the cell surface, can infect cellular debris and can be released from infected cells and be found in plasma. All these caveats notwithstanding, the motile sperm cell fraction resulted to be HPV DNA-negative in all the analyzed samples, suggesting that the washing procedure used in this study is able to yield consistently an HPV-free seminal fraction.

The present study has some limitations, such as the limited number of analyzed samples. However, the vast majority of papers in the literature evaluate the presence of HPV virus in the overall semen, while we successfully analyzed the different fractions. Moreover, the enrolled patients were all considered fertile, according to WHO guidelines (World Health Organization, 2010). This differs from most studies, where males with fertility abnormalities were included.

Our study confirms previous findings that HPV can be detected in semen of HPV infected men and could be common in primary infertile men. Some authors reported that HPV was associated with impaired sperm in particular progressive motility (Boeri et al., 2019). Over all these observations reinforce the idea that HPV screening and diagnosis should be performed in the diagnostic work up of man asking reproductive assistance not only because of its potential pathophysiologic negative impact on male fertility but also for eliminate the virus from the sperm. Thus, as we show that it is possible to obtain HPV-free motile sperm cells, screening all semen for HPV positivity should be considered in every assisted fertility situation (Depuydt* et al., n.d.).

AUTHOR CONTRIBUTION STATEMENT

C.F. and C.V. carried out all the experiments. C.F. wrote the paper with input from all authors. M.O and B.P. collected and processed all the samples, and collected clinical data. D.T., and V.S. supervised the project. S.I. helped in carrying out experiments. A.G. contributed in enrolling subjects. V.S., C.F., D.T., M.B., and M.O. shaped the research. M.C. greatly contributed to the manuscript writing. V.S. conceived the original idea and the overall study. All authors discussed the results and contributed to the final manuscript.

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CONFLICT OF INTEREST

All authors report no competing interest.

FIGURE LEGENDS

Figure 1. Schematic representation of the sperm wash procedure and the five fractions collected for analyses.

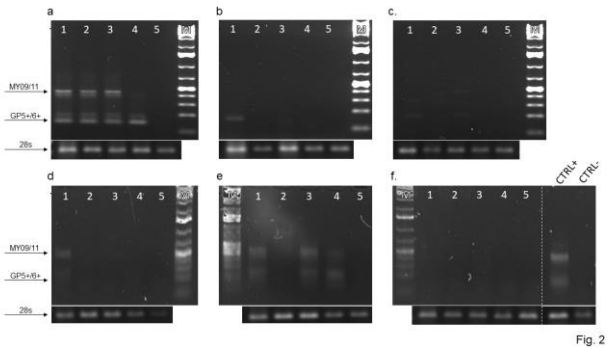

Fig. 2

Figure 2. Amplification results for HPV DNA by nested PCR. Each panel shows results obtained on seminal plasma (1), cell pellet (2), round cells (3), non-mobile sperm cells (4) and mobile sperm cells (5), in addition to 100 bp ladder as size marker (M). Arrows indicates HPV products amplified by MY09/MY11 and GP5+/GP6+ at 450 and 150 bp, respectively. Representative samples resulted HPV DNA positive in at least one of the fractions are depicted in panel a-to-e. A sample resulted HPV negative is depicted in panel f, in addition to a positive and a negative controls. For each fraction, the corresponding amplification of the human gene *28s* is showed below.

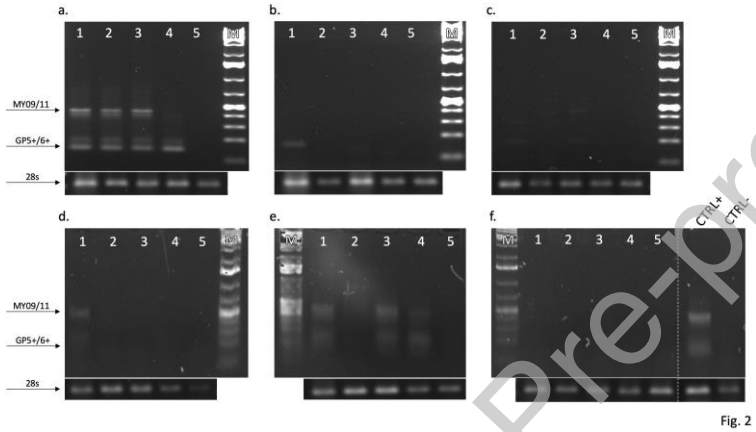

Fig. 2

Table I. Sperm parameters observed in 15 HPV infected patients and negative control. Below the column title, lower yield limit is displayed, as reported by WHO (World Health Organization, 2010).

ID	Semen volume (ml) (1,5 ml)	pH (≥7.2)	Total sperm cells ($39*10^6$)	Sperm cells/ml ($15*10^6$)	Motile sperm cells (%) (40%)	Normal morphology (%) (4%)	Non-motile sperm cells/ml	Swim-up sperm cells/ml	Sperm cells in pellet/ml
1	1	7	$13*10^6$	$13*10^6$	23	1	$0.1*10^6$	$0,05*10^6$	$3*10^6$
2	4.5	7.8	$261*10^6$	$58*10^6$	72	8	$15*10^6$	$10*10^6$	$100*10^6$
3	3	8	$21*10^6$	$7*10^6$	43	3	$0.2*10^6$	$0.4*10^6$	$15*10^6$
4	1.5	8	$126*10^6$	$84*10^6$	52	5	$4*10^6$	$20*10^6$	$120*10^6$
5	2	7.6	$72*10^6$	$36*10^6$	50	2	$6*10^6$	$3*10^6$	$35*10^6$
6	3.5	7.8	$140*10^6$	$40*10^6$	50	15	$3*10^6$	$12*10^6$	$50*10^6$
7	3	7.8	$84*10^6$	$28*10^6$	50	6	$2*10^6$	$5*10^6$	$40*10^6$
8	6	7.4	$26*10^6$	$4.4*10^6$	40	3	$0.1*10^6$	$0.01*10^6$	$6*10^6$
9	2	7.8	$148*10^6$	$74*10^6$	51	16	$5*10^6$	$13*10^6$	$50*10^6$
10	2	7.8	$142*10^6$	$71*10^6$	46	16	$12*10^6$	$10*10^6$	$30*10^6$
11	4.5	7.8	$238.5*10^6$	$53*10^6$	45	11	$6*10^6$	$8*10^6$	$80*10^6$
12	6.5	7.8	$585*10^6$	$90*10^6$	51	5	$10*10^6$	$10*10^6$	$50*10^6$
13	3.5	8	$192*10^6$	$55*10^6$	50	5	$15*10^6$	$0.3*10^6$	$19*10^6$
14	5.5	7.6	$187*10^6$	$34*10^6$	38	7	$5*10^6$	$3*10^6$	$50*10^6$
15	2	7.8	$52*10^6$	$26*10^6$	35	7	$5*10^6$	$3*10^6$	$15*10^6$
CTRL-	2	7.6	$140*10^6$	$52*10^6$	50	26	$1*10^6$	$2*10^6$	$40*10^6$

Table I

Table II. Summary of the nested PCR results showing the number of sperm fractions resulted HPV-negative/positive, as either absolute number or percentage.

N° of patients	Seminal plasma	Cell pellet	Round cells	Non-motile sperm cells	Motile sperm cells
15	10 (66%)	1 (6%)	5 (33%)	3 (20%)	0 (0%)

Table 2

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