Lack of association between HPV and sperm parameters as a risk factor in infertile men admitted to an infertility clinic

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Introduction:
Numerous factors contribute to male infertility including genital infections that may appear following microbial, fungal, and viral infections. Different studies have been recently conducted in the world on papillomavirus infection and its effect on functional parameters of sperm including motility, morphology, sperm count, and reduced male fertility. However, the correlation between papillomavirus infection and male infertility is still ambiguous.

Materials and Methods:
In this study, 50 semen samples of fertile men (as the control group) and 50 semen samples of infertile men were collected from Infertility Center of Qom Jihad Daneshgahi, Qom, Iran. The semen samples were analyzed according to World Health Organization’s standard methods and papillomavirus was detected using PCR and virus L1 region replication.

Results:
The analyzed spermogram of 50 infertile samples showed that 36%, 68%, and 72% of the samples had problems, respectively in terms of count, motility, and morphology, and 52% of the infertile samples had problems in all three parameters. The DNA of human papillomavirus was not detected in any fertile and infertile samples.

Conclusion:
Based on the results of this study, there was no correlation between papillomavirus infection and male infertility. It seems that factors such as male circumcision, lack of homosexual behavior, and lack of multiple sexual partners were effective in reducing the prevalence of HPV in the studied male population.

Keywords: Male Infertility, Papillomavirus, L1 Gene

Introduction
Male infertility is defined as the inability to cause a pregnancy and female infertility is defined as the inability to conceive a pregnancy after one year of intercourse. Also, if a woman fails to maintain the fetus in her uterus and abort her child, she is considered infertile (1). Infertility depends on many factors which are divided into three general categories, including genetic, environmental, and infectious factors. In 1993, the World Health Organization emphasized the role of genital infections in human infertility (2). Most male genital infections may induce infertility. About 15-20% of infertility cases are reported to be caused by semen infection (3). Based on the source of infection, several pathogenic mechanisms have been
explained. Male infertility is caused by bacterial infections of the genitourinary tract. This type of infection in men’s genitourinary tract causes impaired spermatogenesis, and impaired sperm function and structure (motility, viability, acrosome reaction, sperm-oocyte binding, the sperm membrane and DNA damages), and even the genital tract obstruction, and leads to infertility or reduced fertility rate. In addition to bacteria, viruses can also contribute to infertility in men and women (4). Chronic viral infections can infect sperm and are considered a risk factor for male infertility. Studies have shown that infection with human papillomavirus, hepatitis B virus, and hepatitis C virus in the semen impairs semen parameters and reduces sperm motility. Foresta et al. found that infection with human papillomavirus works in a similar mechanism to other viruses to infect sperms. Viral infection on the equatorial region of the sperm head reduces the sperm tail function and the ability of the acrosome, so it will be effective in the binding of gametes (5). Since HPV infection causes fertility problems in men and miscarriage in women, it should be addressed with emphasis on screening, and should be eliminated as one of the risk factors for infertility. With regard to the transmission of this virus and its impact on the fertilized egg, men should be examined in terms of lack of infection using PCR method during in vitro fertilization for infertility treatment in infertility clinics and normal sperm should be selected to increase the efficiency and the possibility of pregnancy. According to the above mentioned, the present study aimed to detect human Papillomavirus (HPV) in men, as a factor contributing to sperm function. Considering that no study has been reported on the prevalence of this virus in male infertility in Iran, the results of this study can be used in infertility clinics for selecting people for in vitro fertilization and sperm donation.

Materials and Methods
In this case-control study, sampling was carried out over a period of six months from March to September, 2013 after obtaining written informed consent from patients aged 25-45 years referring to Infertility Clinic of Qom Jihad Daneshgahi, Qom, Iran. 50 infertile men without varicocele and testicular trauma with one year of unprotected intercourse and without any previous history of uterine and ovarian disorders in their wives (case group) and 50 fertile men having at least one child (control group) were included in the study. Semen samples were collected from patients who had no intercourse in the past 48 hours. The spermogram was performed on all samples according to the World Health Organization standards to examine properties, such as semen volume and PH, liquefacation time, sperm motility, morphology, and count. The collected samples were stored in the freezer at -80 °C for DNA extraction. To extract DNA, a total of 20 µl of semen was first washed with 500 µl of 70% ethanol. Samples were centrifuged at 13000 rpm for 5 minutes and the supernatant was removed. The previous steps were repeated again and after observing the white deposit, 500 µl of lysis buffer and 2.5 µl of Triton-X100 (0.5%), 21 µl of DDT (1M) and 40 µl of proteinase K (10 mg/ml) were added. Samples were mixed well with digestion buffer and were incubated at 50 °C for one day. The microtubes were then centrifuged at 15000 rpm for 10 min and the supernatant was removed. The previous steps were repeated again and after observing the white deposit, 500 µl of lysis buffer and 2.5 µl of Triton-X100 (0.5%), 21 µl of DDT (1M) and 40 µl of proteinase K (10 mg/ml) were added. Samples were mixed well with digestion buffer and were incubated at 50 °C for one day. The microtubes were then centrifuged at 15000 rpm for 10 min and the supernatant was transferred to new micro-tubes. Then, 1 µl of glycogen (20 mg/ml) and one-tenth volume of NaAc (3M) were added to samples and mixed vigorously. Two volumes of cold absolute ethanol was then added to samples and they were kept at -80 °C for 1-2 hours for DNA deposition. The resulting substance was centrifuged at 13000 rpm for 20 minutes and the deposit was extracted by discarding the supernatant. The resulting DNA deposit was washed in 500 µl of 75% ethanol and
centrifuged at 13000 rpm at room temperature for 10 minutes and then dried. Finally, the deposit was dissolved in TE buffer and its concentration and purity were assessed by Nanodrop (6).

**Beta-globin polymerase chain reaction (PCR):**
Polymerase chain reaction was performed in a final volume of 25 µl, including 12.5 µl of Amplicon buffer, 0.5 µl (0.4 mM) of Beta globin gene-specific primer GH20 (5’ GAA GAG CCA AGG ACA GGT AC 3’), PC04 (5’ CAA CTT CAT CCA CGT TCA CC 3’), and 50 ng of the extracted DNA to prove DNA extraction from samples. DNA amplification was carried out using a thermocycler (Bio Rad PCR System, USA) according to the temperature program of the initial denaturation step at 95°C for 3 minutes, 45 primer binding cycles of 95°C for 30 sec, 53°C for 40 sec, and 72°C for 40 sec. At the end, the primer extension step was conducted at 72°C for 5 min. The PCR products were qualitatively examined on 2% agarose gel in an electrophoresis tank containing TBE buffer (Tris Base, Boric acid, Na EDTA, Deionized Water) at 100 V for an hour and the results were analyzed by a GelDoc device.

**HPV L1-specific PCR:**
MY09/MY11 primers were used for L1-specific PCR (7). MY09: CGT CCM ARR GGA WAC TGA TC and MY11: GCM CAG GGW CAT AAY AAT GG. DNA amplification stages, the final primer extension, and qualitative study of the resulting product were conducted similar to the previous case.

**Results**

**Quantitative analysis of DNA extracted from semen samples:**
Semen, also known as seminal fluid or seminal plasma, contains spermatozoa, fructose and various proteins that are essential for the survival of the spermatozoa, but may reduce the purity and quality of extracted DNA. Therefore, it must be washed with ethanol before extracting DNA (6). Spermatozoa are protected by a membrane rich in disulfide bonds interfering in the process of DNA extraction and cell lysis. A powerful antioxidant, dithiothreitol, should be used to break these bonds, make sperm cells more sensitive and permeable to digestion buffer and protein kinase K activity, and facilitate DNA extraction. By binding to proteins, sodium dodecyl sulfate forms an insoluble complex, the upper phase (containing nucleic acids) of which precipitates during centrifuge and can be easily transferred to new microtubes. Given that the role of EDTA is to stop Dnase activity and both glycogen and sodium acetate are attached to nucleic acids, it helps the DNA deposition. Both of these substances must be added to the sample before adding ethanol. To ensure that the DNA was extracted from sperm samples, beta-globin PCR was also performed in addition to Nanodrop assessment. The quantitative analysis of DNA extracted from samples using a spectrophotometer (nanodrop) indicated the proper absorption of DNA solution at the wavelength of 260 nm (ranged from 1.7 to 2). It indicated that the DNA extracted from samples was suitable for PCR amplification. The spermiogram of 100 samples indicated a normal analysis in 50 samples as controls and an abnormal analysis in some parameters of sperm in 50 patients. Sperm motility, count, and morphology are among the most important factors in evaluating sperm quality and infertility which were reported 68%, 46%, and 72% respectively less than the standard levels, so they were considered infertile. Of the 50 infertile samples, 52% had problems in all three factors, i.e., count, morphology, and motility.

**The results of the beta-globin gene amplification:**
In addition to Nanodrop assessment, the beta-globin PCR was also conducted on
100 samples in which the amplification of the 268bp fragment in all samples was indicative of high quality of the extracted DNA. All samples were amplified by beta-globin-specific primers and the 268bp product was confirmed on agarose gel, which resulted from the confirmation of the DNA extracted from samples. Agarose gel profile is shown in Figure 1.

**The results of HPV L1 amplification:**
After performing PCR with L1-specific primers, 450Bp indicating the amplification of this fragment was not observed in samples. PCR was performed in positive control samples and the mentioned fragment was amplified only in positive controls and was not observed in fertile and infertile samples. Therefore, all samples were negative for HPV infection (Figure 2).

![Figure 1: The beta-globin PCR as an internal control for assessing DNA extraction. Wells 1-14: Beta globin gene PCR product (268bp fragment) in a number of fertile and infertile samples, 100 base pairs DNA Ladder: M, c-: negative control.](image1)

![Figure 2: HPV L1-specific PCR profile (450 bp fragment) on 2% agarose gel. Wells 1-5: lack of amplification of 450 bp fragment in a number of fertile samples, well 6: positive control, well M: 100 base pairs DNA Ladder, well 7: positive control, well 8: negative control, wells 9-15: lack of amplification of 450 bp fragment in a number of infertile samples.](image2)

**Discussion and Conclusion**
Human papillomavirus is one of the most important viruses that transmit sexually. So far, more than 90 genotypes of this virus have been identified that infect the genital area, at least 13 genotypes of which are considered high-risk or oncogenic (8). HPV infection is prevalent in sexually active men’s sperm and the hypothesis of the relationship between HPV infection and male infertility has already been raised (9). Several studies have been conducted on the relationship between infection with this virus, changes in the quality of sperm parameters and the incidence of infertility, but this correlation has not certainly been confirmed. In fact, some studies have suggested the negative impact of this virus on sperm parameters, and some others have reported no relationship between...
infection with this virus and changes in the quality of sperm parameters and even abortion.

Bezold revealed that semen infections, including HPV infection also exist in asymptomatic men and are often associated with low quality of sperm and changes in two main factors, i.e. the progressive motility and sperm morphology that ultimately affect male fertility and can even lead to infertility (10). Several studies have also reported the reduced sperm motility as a result of infection with HPV (9, 11, 12, 13, 14).

According to a study conducted by Yang et al. on fertile and infertile groups, it was found that HPV infection is more prevalent in infertile men compared to fertile men. In addition, the progressive motility and normal morphology of sperm clearly decrease in infected individuals (15). According to a study by Cai et al. (2014) on the effect of simultaneous HPV and Chlamydia trachomatis infection on sperm parameters, including concentration, motility, and morphology, it was reported that simultaneous HPV and Chlamydia trachomatis infection decreases the quality of sperm parameters, particularly motility and morphology (16). In contrast, some other studies have reported no relationship between HPV infection and the quality of sperm parameters. Tanaka et al. (2000) also conducted a study on the prevalence of HPV type 16 among couples undergoing IVF and its impact on sperm parameters and reported no correlation between this virus and the quality of sperm motility. They believed that HPV infection has no effect on abortion and IVF failure (17). According to another study conducted by Foresta et al. (2010) on the evaluation of sperm infection with HPV and its effect on sperm parameters in two groups with and without sexual history, it was reported that HPV was present in the group with a history of unprotected sex and infection was associated with reduced sperm motility, while no HPV infection was observed in the group without sexual history (18).

By conducting a study on men whose wives were undergoing IVF, Schillaci et al. found that HPV infection existed in 7.8% of their semen. It should be noted that oncogenic HPV genotypes were confirmed in asymptomatic subjects, but the role of infection in infertility was not confirmed (19). This hypothesis that the infected sperm may lose HPV infection during washing is not accepted, because in a study conducted on infertile patients infected with this virus, it was proved that washing has no effect on the elimination of this virus (20). The prevalence of HPV ranges from 0% to 73% across the world. Several studies have shown that, on average, more than 50% of the men and women who are sexually active will have HPV infection in their lives and the risk of this infection can increase by having multiple sexual partners, unprotected sex, and smoking. HPV infection and its genotype distribution have rarely been reported among Asian men, especially men whose fertility has been proven (15). Circumcision may reduce the risk of HPV infection in men and the transmission of HPV to their female partners, which would reduce the risk of cervical cancer in women. Male circumcision reduces the contact surface of the penis and prevents phimosis. Reduced contact area of the penis is one of the factors reducing the risk of HPV infection. A study conducted in the United States reported that the prevalence of HPV infection in the glans penis (corona) as 46% in uncircumcised men and 29% in circumcised men (8). In addition, homosexual behavior and having multiple sexual partners increase the risk of HPV infection (15). In this study, PCR method was used, thus it is likely that the HPV genomes was detected with high sensitivity. In this method, general primer sequences that have been conserved among a wide range of HPV types were used to detect HPV types in samples. Detergent primers used in this study were
able to identify high-risk and low-risk genotypes. The method used in this study was absolutely valid and this method has also been used in other studies conducted in other countries to detect certain types of viruses. In the present study, HPV types were first detected with general primers and by running the amplified products on agarose gel, the intended band was not observed except for the positive controls. The results may be changed if the number of samples studied increases. Iran is an Asian country and the prevalence of this virus on this continent is less than other continents. In our Islamic country, almost all men are circumcised in early childhood which is another reason for reduced prevalence of HPV in Iran. Homosexual behavior has been reported as another reason for the prevalence of this virus. Homosexual behavior in developed countries is seen much more than in our country and this is another reason why HPV is less prevalent in Iran. Having multiple sexual partners is among the main causes of HPV prevalence in the world. Due to religious beliefs, this behavior is less common in Iran compared to developed countries. In addition, samples were collected from married people who lived in the religious city of Qom and had sexual intercourse only with their married partners. It was another reason for decreased HPV prevalence in the region studied (Qom). Therefore, no HPV infection observed in the 100 samples examined cannot be a conclusive reason for the lack of effect of HPV infection on male infertility; because in studies conducted around the world, the relationship between HPV infection and male infertility has been confirmed. The prevalence of this virus depends on the geographical region, race, socioeconomic status, and the frequency of sex, as well. The result of this study is similar to the study of Foresta (2010) in which HPV was not observed in people with no history of sexual intercourse (13). They reported no HPV infection in their study population, which can be attributed to factors such as circumcision, marriage, commitment to ethical principles, and lack of multiple sexual partners. Given that all participants were negative for HPV infection in this study, it can be said with certainty that changes in sperm parameters, including sperm morphology, motility, and count were not associated with HPV infection and other factors, such as bacterial and viral infections should be examined. The results of this study are consistent with other studies that have reported changes in sperm parameters are independent from HPV infection.

It is suggested that the semen analysis test be conducted on infertile men with risk factors (genital warts, positive partners, and previous infections) in terms of the presence of papillomavirus. Given that HPV is also effective in spontaneous abortions, in such cases, the semen can be examined in terms of the presence of this virus and if positive, patients can receive an appropriate treatment to make their sperm free from the virus. It is also suggested that the presence of virus be examined in larger populations and different geographic regions using other accurate methods, such as the real-time PCR, and the screening test be performed on men in terms of the presence of the virus.

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Conflict of Interest
The authors declare no conflicts of interest regarding the compilation/ publication of this article.
References:


