

Detection of Herpes Simplex Virus in First Trimester Pregnancy Loss Using Molecular Techniques

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Abstract. *Background: The etiology of early pregnancy loss is multifactorial and may include viral pathogens. The aim of this study was to investigate the presence of herpes simplex virus (HSV) in gestational tissues of first trimester pregnancy loss using molecular techniques. Patients and Methods: Gestational tissue from 95 women with first trimester pregnancy loss and 36 women with elective termination of pregnancy was investigated by nested polymerase chain reaction (PCR) and in situ hybridization techniques. Results: HSV was detected by the nested PCR technique in 41 out of the 95 (43.2%) cases of early pregnancy loss and in 6 out of the 36 (16.7%) cases of elective pregnancy termination ($p=0.03$). The HSV-positive cases in the early pregnancy loss group were observed between the 6th and the 12th gestational week and mostly in the 9th, 8th and 6th week. The in situ hybridization technique was successful in 18 out of 25 PCR-positive cases (72%) and revealed that the hybridization signal was mostly localized in the nuclei of the decidual and intermediate trophoblastic cells and less commonly in the chorionic villous trophoblastic cells. Conclusion: HSV seems to play a significant role in first trimester pregnancy loss and its detection by sensitive and accurate molecular techniques would permit prompt antiviral therapy for a successful future pregnancy.*

First trimester pregnancy loss is a very common problem occurring in more than 50% of all pregnancies, with only 15-20% of all cases being clinically recognized (1). The etiology of early pregnancy loss is not clearly understood and may include more than one cause. The most common etiological factors are genetic abnormalities, autoimmune and endocrine disorders, anatomic uterine defects and finally infections (2, 3).

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Primary or recurrent herpes simplex virus (HSV) infection in pregnancy and its serious consequences for the fetus and neonate have attracted much interest. Specifically, primary HSV infection during the second or third trimester can be related to pre-term labor, fetal abnormalities and pregnancy loss, whereas recurrent HSV infection constitutes a much lower risk for the embryo, fetus and neonate (4-6). In contrast, the association of HSV with first trimester pregnancy loss, despite the initial epidemiological observations (7-9), has not been fully elucidated and remains controversial. Consequently, we found it of great interest to investigate the presence of HSV in gestational tissues of first trimester pregnancy loss utilizing the sensitivity of polymerase chain reaction (PCR) and the localizing ability of *in situ* hybridization molecular techniques.

Patients and Methods

Ninety-five cases of women, aged 25-44 years, with first trimester pregnancy loss treated by endometrial suction curettage in the Mitera General, Maternity and Children Hospital of Athens were included in the study. Sixty-five of these women had no history of previous pregnancy loss, whereas 24 reported one and 6, two previous pregnancy losses in their history. Eighteen of the women had had a previous live birth. None of the women or their husbands reported any clinically confirmed genital herpes in their medical history. None of the women was immunocompromised. Gestational tissues from 36 women with elective abortion were used as a control group. A portion of the gestational tissue from all the cases was snap frozen and stored at -70°C until the time of molecular analysis. The remaining tissue was fixed in neutral buffered formalin and embedded in paraffin for histological examination and *in situ* hybridization. Informed consent was obtained from all the women who participated in this study.

Polymerase chain reaction. PCR detection of HSV was performed on DNA extracted from frozen gestational tissue samples using the protocol of the Epicentre DNA extraction kit (Epicentre Biotechnologies, Madison, WI, USA). All the samples were examined for the presence of HSV DNA by the nested PCR technique using one set of outer primers for primary PCR and one set of inner primers for nested PCR (Table I). Initially, 3-5 μl extracted DNA from each sample were amplified in a reaction solution of 50 μl containing 25 mM MgCl_2 , 0.25 μM of each

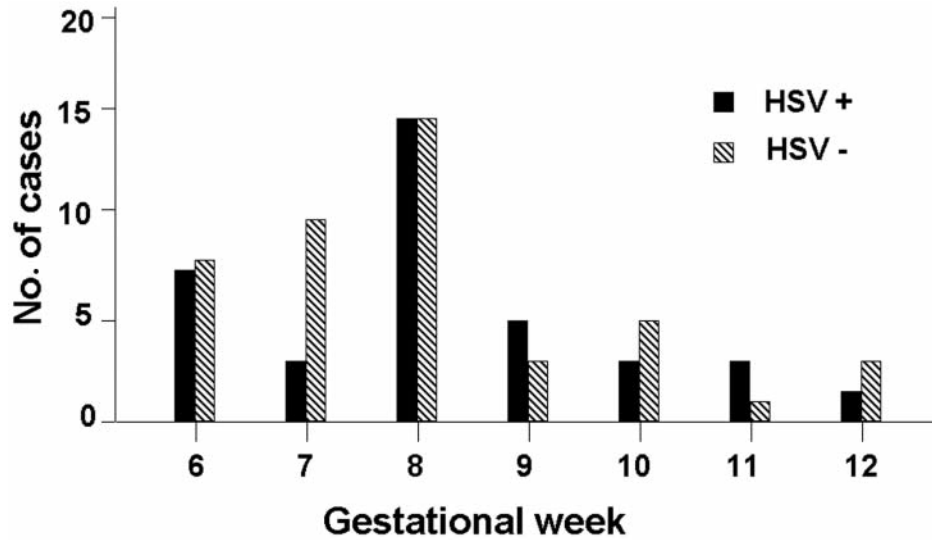


Figure 1. Distribution of HSV-positive and -negative cases according to gestational week.

Table I. Primers used in the detection of HSV in the gestational tissue samples.

HSV Gene	Sequence	PCR product
HSV-1 DNA pol gene (UL30 gene)	Outer Primers 5-CCC GTG GTG GTG TTC GAC TTT GCC-3 5-GCA CAA AGA TGG AGT CCG TGT CCC-3	544 bp
	Inner primers 5-TTC TTC GTC AAG GCT CAC GTG CG-3 5-CCG AGT TAC ACA CGA CCT TGA TGG C-3	154 bp

primer, 200 µM of each dNTP, 1x PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 22°C) and 2.5 U Taq Polymerase (Epicentre MasterAmp Taq DNA polymerase). Subsequently, the same reaction mixtures were applied for the nested PCR using 2 µl of the primary PCR product as a template.

The primary PCR mixture was heated for 5 minutes at 95°C and then subjected to 40 cycles of amplification in the following conditions: denaturation at 95°C for 45 s, annealing at 60°C for 45 s, elongation at 72°C for 1 min. For the nested PCR the mixture was heated for 5 minutes at 95°C and then subjected to 40 cycles of amplification in the following conditions: 95° for 45 s, 57°C for 45 s and 72°C for 45 s. The primary and nested PCR reactions ended with an elongation step at 72° C for 5 minutes.

DNA extracted from HSV-1 and HSV-2 infected Vero cells (ATCC, Manassas, Virginia, USA) was used as positive control, and a reaction mixture devoid of template DNA as negative control. The PCR products were examined by electrophoresis in a 2% agarose gel and photographed on a UV light transilluminator.

Comparison of the HSV incidence between the pregnancy loss and the control group cases was performed using the Fisher exact test. A *p*-value lower than 0.05 was accepted as statistically significant.

In situ hybridization. The *in situ* hybridization technique was applied to the formalin fixed paraffin embedded gestational tissue sections from 25 PCR-positive cases in order to study the morphological localization of the HSV DNA. Briefly, the technique was applied as follows.

The sections were deparaffinized and digested with 250 µg/ml proteinase K (AppliChem, Darmstadt, Germany) on a 37°C heating plate for 15 min, washed in PBS, dehydrated in graded ethanols and air dried. A HSV (1, 2) DNA probe labeled with digoxigenin (PanPath, Budel, the Netherlands) was applied to the section and covered with a plastic coverslip. Subsequently, denaturation of the probe and the target DNA was simultaneously performed on a 95°C hot plate. The hybridization reaction was performed by incubating the slides in a humid chamber at 37°C for 16 hours. Post hybridization washing of the specimens was performed in 1xSSC/50% formamide (1xSSC=0.15 M NaCl, 0.015 M sodium citrate) at 42°C for 15 min. The hybridization reaction was detected using antidigoxigenin-alkaline phosphatase at room temperature (RT) for 30 min and bromochloroindolyl phosphate/nitroblue tetrazolium (BCIP/NBT) chromogenic substrate at RT for 40 min. Finally, the sections were counterstained with nuclear fast red, dehydrated and mounted. Slides containing HSV infected and uninfected fibroblasts served as positive and negative controls respectively.

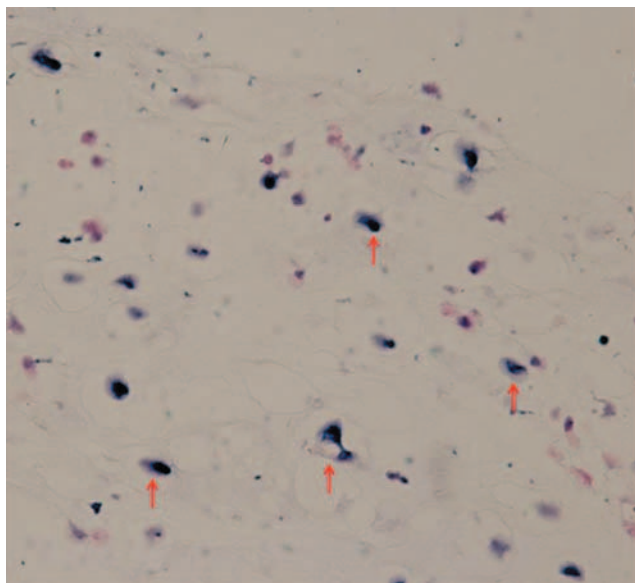


Figure 2. HSV detection in gestational tissue of a case of pregnancy loss by *in situ* hybridization. HSV DNA is localized in the nuclei of decidual and intermediate trophoblastic cells (purple, arrows). Original magnification $\times 200$.

Results

In the cases of first trimester pregnancy loss, HSV was detected by the nested PCR technique in 41 out of the 95 (43.2%) gestational tissue samples. By the first PCR, only 3 cases were found to be HSV positive, whereas the viral culture control samples were positive by both first and second PCR. The PCR method used was found to be unable to discriminate between HSV1 and HSV2, as both viral cultures were strongly positive using the HSV1 DNA polymerase gene primers. The HSV positive cases were observed between the 6th and the 12th gestational week and mostly in the 11th, 9th, 8th and 6th week (Figure 1). In the elective abortions control group, HSV was detected by the nested PCR technique in 6 out of the 36 cases (16.7%). The difference of HSV incidence between the cases of first trimester pregnancy loss group and the elective abortions control group was found to be statistically significant ($p=0.03$).

By the *in situ* hybridization technique, HSV DNA was detected in 18 out of the 25 (72%) PCR-positive cases and was localized in the nuclei of the decidual and intermediate trophoblastic cells and less commonly in the chorionic villous cytotrophoblast and syncytiotrophoblast cells (Figure 2 and Figure 3). Microscopic evaluation of the HE-stained specimens showed signs of viral cytopathic effects in the form of intranuclear inclusions and multinucleated giant cells only in two cases.

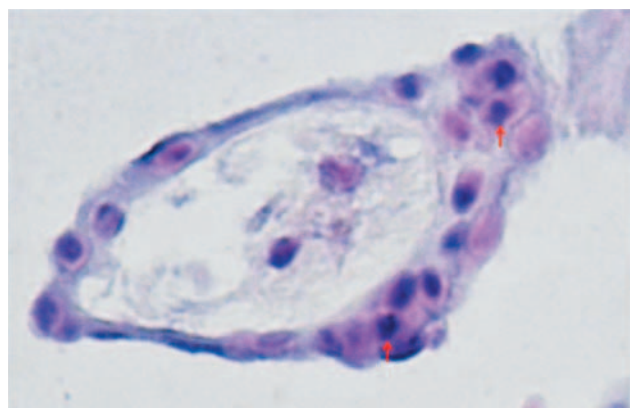


Figure 3. *In situ* hybridization application in another case of pregnancy loss showing a chorionic villous in which HSV DNA is localized in the nuclei of cytotrophoblast and syncytiotrophoblast cells (purple, arrows). Original magnification $\times 400$.

Discussion

According to our knowledge, this is the first report of such a significant incidence of HSV infection in gestational tissues from immunocompetent women with early pregnancy loss is reported. In a previous publication concerning HIV-positive women (10), HSV was found to be detected in 72% of the examined gestational tissue samples by the *in situ* hybridization technique. In another study, HSV was detected in only 3 out of 102 examined cases of aborted material using a simple PCR technique (11). It may be possible that the quantity or the physical status of viral DNA in the gestational tissue of pregnancy loss does not permit its easy detection and requires the use of the more sensitive and specific nested PCR technique. In the present study, the *in situ* hybridization technique showed 84% HSV detection sensitivity in relation to nested PCR and thus can be considered as a very good alternative for the detection of this virus when paraffin-embedded material is available.

The localization of HSV DNA in the nuclei of decidual cells indicated that it may represent a reactivation of a preexisting endometrial infection due to temporal attenuation of natural killer cell cytotoxicity in early pregnancy (12). An endometrial pool of HSV can be reasonably accepted considering the fact that male sperm, which is infected by HSV in a noticeable number of cases (13-15), can easily transmit the virus to the endometrium during sexual intercourse. However, the prevalence of HSV-related endometrial infections, as well as that of genital herpes are underestimated because many of these cases are misdiagnosed or unrecognized (16). In contrast to currently prevailing beliefs, El Borai *et al.* detected HSV DNA in menstrual blood by PCR in more than half of the infertile women examined in their study (13).

The mechanism by which HSV infection is involved in pregnancy loss is a matter of hypothesis. Reactivated endometrial HSV infection may be associated with a subsequent increase in natural killer cell activity which has been found to be related to pregnancy loss (17, 18), or it may cause dysregulation of the Th1 to Th2 cytokine shift mechanism (19). HSV may also be linked with enhanced apoptosis in decidual and trophoblastic tissues, which has been suggested as a possible mechanism of spontaneous pregnancy loss (20, 21). Moreover, a thrombogenic action caused by HSV on the uteroplacental vessels leading to circulatory disturbance cannot be ruled out as a mechanism of spontaneous pregnancy loss, given the fact that HSV as well as cytomegalovirus (CMV) has the ability to cause trombin production and endothelial damage (22).

In conclusion, HSV may play a significant role in first trimester pregnancy loss and its detection by sensitive molecular techniques would allow prompt therapeutic intervention in order to increase the possibility of a successful future pregnancy.

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