Investigation of Frequency of Herpes Simplex Virus in Patients with Type 2 Diabetes and Healthy Individuals by PCR and ELISA

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ABSTRACT

Background and Objectives: Previous studies have demonstrated the relationship between viral infections and risk of developing type 1 diabetes. The aim of this study was to investigate the frequency of Herpes simplex virus (HSV) in patients with type 2 diabetes and healthy control individuals using PCR and ELISA.

Methods: Blood samples were taken from 180 diabetic patients and 187 healthy controls referred to the Pasteur medical laboratory in Tonekabon, in 2016. Human beta-globin gene was used as internal control to ensure extraction accuracy. Specific primers were used for amplification of the *UL30* gene. In addition, level of anti-HSV IgG antibody was measured using a commercial ELISA kit (Euroimmun, Germany).

Results: DNA of HSV was found in the samples of 11 patients (6.1%) and five healthy controls (2.7%). In addition, anti-HSV IgG was found in the samples of 117 patients (65%) and 108 healthy controls (57.75%). There was a statistically significant relationship between frequency of anti-HSV IgG and diabetes.

Conclusion: Similar to previous studies, the present study demonstrated a relationship between frequency of HSV infection and type 2 diabetes. However, further studies should be performed to eliminate the effect of other risk factors to help clarify the exact role of viral infections in increasing the risk of diabetes.

Keywords: Diabetes, Herpes Simplex Virus, ELISA, PCR.

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INTRODUCTION

Diabetes refers to a group of metabolic disorders characterized by chronic hyperglycemia. Considering the rising prevalence of diabetes worldwide, it is expected that the disease will remain as one of the main causes of morbidity and mortality (1). Several factors such as genetic susceptibility, obesity, age, high blood pressure, high blood lipids, and several environmental factors are associated with incidence of diabetes (2). Determining the initial environmental factor is not simple because the exposure to the factor might have occurred many years before development of diabetes. A possible environmental factor is infection with different families of viruses (3-10). *Herpesviridae* is a large family of viruses that are pathogenic to humans. Identifying characteristics of these viruses, helps clarify their ability in causing latent infections (11). Herpes simplex virus (HSV) is widely distributed throughout the world. These viruses have various hosts and are capable of reproducing in different types of cells. HSV can cause several diseases, including chronic gingivostomatitis, keratoconjunctivitis, encephalitis, genital disease, neonatal infection, and latent infection of neurons (11).

The aim of this study was to investigate prevalence of HSV in patients with type 2 diabetes and healthy controls using polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA).

MATERIAL AND METHODS

This randomized cross-sectional study was conducted on 180 diabetic patients and 187 healthy blood donors (control group) referred to the Pasteur medical laboratory in Tonekabon, in 2016. Diabetes was confirmed in the participants via laboratory testing and by an internal medicine physician. Blood samples were taken from the participants after obtaining written consent.

Effect of any underlying disorder including immunodeficiency, allergy, cancer, etc. was eliminated. After recording demographic information of the participants, 5 ml blood samples were taken and transferred to tubes containing EDTA. After centrifuging the samples, 50 µl of plasma was transferred to another sterile tube in order to perform ELISA. The remaining samples were stored at -20 ^{o}C until DNA extraction process.

DNA extraction was carried out using commercial kits (Qiagene, Lot No: 11872534, Cat No: 51306) according to the manufacturer's instructions. Purity of the extracted DNA was analyzed by assessing absorbance at 260 and 280 nm using a BioPhotometer (Eppendorf, Germany).

Human beta-globin gene was co-amplified with the target fragment, as an internal amplification control, using the following primers: Forward: 5'-TCC AAC ATC AAC ATC TTG GT-3' and Reverse: 5'- TCC CCC AAA TTC TAA GCA GA-3' (12).

The following specific primers were used for amplification of the *UL30* gene: Forward: 5'-CAG TAC GGC CCC GAG TTC GTG A -3' and Reverse: 5'- GTA GTA GGT GCG GGT GAT GTT -3' (13). The primers were synthesized by TAG Copenhagen (Denmark).

PCR was performed in a 25 µl reaction mixture containing 13 µl of molecular biology-grade water (Sigma Aldrich Company LTD., USA), 2.5 µl of 10× PCR buffer (Promega, USA), 1 µl of forward and reverse primers, 1 µl of 10 mM dNTPs (Promega, USA), 0.5 µl of smart taq DNA polymerase (Promega, USA), 1 µl of 50 mM $MgCl_2$ (Promega, USA), and 5 µl of DNA template. The negative control contained all PCR reagents and 5 µl of water instead of the DNA template.

A small aliquot of PCR products was electrophoresed on 1.5% (w/v) agarose gel stained with ethidium bromide at 80 V for 60 min. Results of the electrophoresis were visualized and photographed under a UV-transilluminator (UV doc, England).

ELISA was performed to measure the level of anti-HSV IgG using commercial kits (Euroimmun, Germany), according to the manufacturer's instructions. First, samples and the ELISA kit were placed at room temperature for 30 minutes. A 1:10 dilution of plasma and dilution buffer was prepared. Then, 100 µl of the standards 1, 2 and 3, as well as positive and negative controls and plasma samples were poured into the microplate. The plate was incubated at room temperature for 30 minutes. Each well was washed three times with 450 µl of buffer solution. Then, 100 µl of enzyme conjugate were added to each well. The plate was incubated at room temperature for 30 minutes and the washing was repeated. Next, 100 µl of

the substrate/choromogen solution was added to each well.

After 15 minutes of incubation in darkness at room temperature, 100 µl of stop solution was added to each well. Finally, absorbance was measured at 450 nm and 630nm in an ELISA microplate reader (Germany).

Data analysis was done in SPSS (version 17). Chi square test was used to compare the results at significance of 0.05.

Table 1– PCR conditions for amplification of *human beta globin* and *UL30* genes

Steps	Human beta globin gene		UL30 gene	
	Temperature and duration	Cycle	Temperature and duration	Cycle
Initial denaturation	95 °C - 5 min	1	94 °C - 10 min	1
Denaturation	95 °C - 45 sec	35	94 °C - 60 sec	35
Annealing	54 °C - 30 sec		63 °C - 30 sec	
Extension	72 °C - 30 sec		72 °C - 50 sec	
Final Extension	72 °C - 10 min	1	72 °C - 10 min	1

RESULTS

Detection of the 122 bp and 480 bp fragments indicated presence of the Human beta globin gene and *UL30* gene, respectively (Figures 1 and 2). All samples were positive for beta globin gene, which shows the accuracy of DNA extraction.

The samples of 11 diabetic patients (6.1%) and five healthy controls (2.7%) contained the HSV DNA. Table 1 shows the frequency distribution of HSV DNA based on gender and age group. The results showed that 4.8% of men and 7.3% of women in the diabetic group and 1.1% of men and 4.1% of women in the control group had been infected with HSV. Moreover, 10% of the patients under 40 years, 6% of the patients aged 40-60 years, and 2.5% of the patients older than 60 years were positive for infection with the virus. Furthermore, 4.5% of the healthy individuals aged under 40 years and 2.8% of the controls aged 40-60 years were positive for infection with the virus.

Figure 1- Gel electrophoresis of Human beta-globin amplification products on 1.5% agarose gel stained with ethidium bromide. The 122-bp band (columns 1 and 2) indicates the presence of beta globin gene. Column M: 100 bp DNA ladder, Column 3: Negative control.

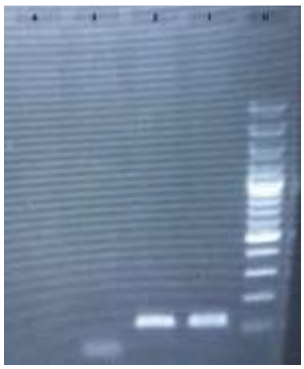

Figure 2- Gel electrophoresis of HSV gene amplification products on 1.5% agarose gel stained with ethidium bromide. Detection of the 480 bp band indicates the presence of HSV DNA. Column M: 100 bp DNA ladder, Column 1-4: amplified HSV DNA, Column 5: Negative control.

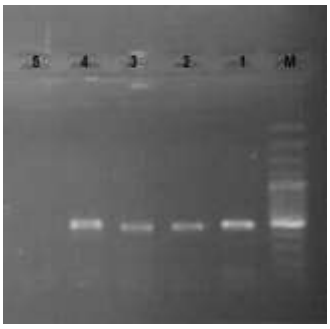

Table 1- Frequency distribution of HSV DNA based on gender and age group

Demographic variable	Status	Patients group				Control group			
		No. Tested	No. HSV Positive	(%)	P Value	No. Tested	No. HSV Positive	(%)	P Value
Sex	Men	84	4	4.8%	0.48	90	1	1.1%	0.37
	Women	96	7	7.3%		97	4	4.1%	
Age (years)	< 40	40	4	10%	0.393	45	2	4.5%	0.21
	40 - 60	100	6	6%		110	3	2.8%	
	> 60	40	1	2.5%		32	0	0	

Table 2- Frequency distribution of anti-HSV antibody based on gender and age

Demographic variable	Status	Patients group				Control group			
		No. Tested	No. HSV Positive	(%)	P Value	No. Tested	No. HSV Positive	(%)	P Value
Sex	Men	84	43	51.2%	0.001	90	32	35.5%	0.001
	Women	96	74	77%		97	76	78.3%	
Age (years)	< 40	40	35	87.5%	0.001	45	29	64.5%	0.001
	40 - 60	100	74	74%		110	74	67.3%	
	> 60	40	8	20%		32	5	15.6%	

There was no significant correlation between frequency of HSV and demographic variables in the participants of both groups.

Based on the results obtained from the ELISA test, 117 patients (65%) and 108 healthy controls (57.75%) had the anti-virus specific antibody. In addition, 51.2% of men and 77% of women in the patient group as well as 35.5% of men and 78.3% of women in the control group had the anti-virus specific antibody. Moreover, the samples of 87.5% of the patients aged under 40 years, 74% of the patients aged between 40 and 60 years and 20% of the patients older than 60 years were positive for infection with this virus. As shown in Table 2, there was a significant relationship between frequency of anti-HSV antibody with gender and age of the participants.

DISCUSSION

The prevalence of diabetes has increased notably in the past few decades. It is expected that the disease will affect more than 438 million individuals by 2030 (14). In Iran, diabetes is the most prevalent non-contagious disease with prevalence of 5.5% (15). Several studies have investigated the frequency of viral infections among diabetic patients and healthy individuals. The results of these studies confirmed the potential role of infection with some viruses including Parvovirus, Coxsackie B, Adenovirus, Rubella, Cytomegalovirus, and HSV in development of diabetes (1-5).

The frequency of HSV in diabetics and healthy individuals was determined in this study. The prevalence of the virus in diabetics and healthy controls was 6.1% and 2.7%, respectively. Frequency of anti-HSV IgG in diabetic patients and healthy controls was 65% and 57.75%, respectively. In line with our study, a study conducted by Sun et al. showed that the frequency of HSV DNA and IgG against the virus was higher in type 2 diabetes patients compared with healthy controls (16). A study by Pak et al. confirmed the relationship between infection with human herpesvirus-5 and the risk of developing type 1 diabetes (17). Several studies have reported the higher frequency of HSV and anti-HSV IgG in patients with type two diabetes compared with healthy individuals. These results suggest a relationship between infection with the virus and the risk of developing type 2 diabetes. Some studies indicated that infection with HSV might increase susceptibility to diabetes via damage to the pancreatic cells (4). Studies of Jia and Guo, Nordal et al., Roberts and Cech, and Chen et al. demonstrated that infection with human herpesvirus-5 increases the risk of developing diabetes via direct pancreatic cell damage and disruption of insulin release from pancreatic beta cells (18-21). Detecting the genetic material of the virus confirmed presence of the virus in diabetic patients. Therefore, preventing infection with the virus could help reduce the risk of

developing type 2 diabetes. On the other hand, diabetic patients are more susceptible to viral infections because of their weakened immune system (21).

CONCLUSION

Similar to previous studies, the present study demonstrated the relationship between frequency of HSV infection and type 2 diabetes. However, further studies should be performed to eliminate the effect of other risk factors to help clarify the exact role of viral infections in increasing the risk of diabetes.

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CONFLICT OF INTEREST

There is no conflict of interest.

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