Keywords: Serotonin receptor (5-HT\textsubscript{3RA}); Monoamine oxidase (MAO-A); Gene expression; Multiple sclerosis

Introduction

Multiple Sclerosis is a common disease of central nervous system associated with multifocal destruction of myelin sheath in neurons, which leads to gradual neuronal damage due to autoimmune response against self-antigens in neurons of genetically predisposed individuals. The disease usually begins between 22 and 42 years of age and is more prevalent in women than men [1-4]. T-cells play an important role in organizing the mechanism of cascade complex in MS, which involves chronic inflammation, initial demyelination and axonal damage [5]. Neurobiology and immunology studies indicate that immune system regulation is affected by external factors in the nervous system known as neurotransmitters, including serotonin or 5-hydroxytryptamine (5-HT) [6]. Evidence has shown that fluctuation of serotonin level can be effective in progress of MS [7,8]. Seven families of serotonin receptors (5-HT\textsubscript{i,j,k}) have been identified. All the receptor families (except for 5-HT3) are G-Protein Coupled Receptor (GPCR), while 5-HT belongs to ligand gated ion channel family (Cys-loop type). Nine exons in its gene make up the protein-coding region. Among the subunits of serotonin (5-HT\textsubscript{1A}, 5-HT\textsubscript{2A}, 5-HT\textsubscript{2C}, 5-HT\textsubscript{3A}, 5-HT\textsubscript{3C}, 5-HT\textsubscript{3D}, 5-HT\textsubscript{3E}, 5-HT\textsubscript{3F}) receptors, 5-HT\textsubscript{3A} is the crucial subunit in the function of receptor [9,10].

Evidence has indicated the presence of 5-HT\textsubscript{3A} receptors on immune cells, including dendritic cells, monocytes, macrophages, T-cells and B-cells [11-14]. Serotonin binding to 5-HT\textsubscript{3A} receptor causes the influx of calcium ions into cells along with other input and output ions [15]. Influx of calcium ions into T-cells is one way to activate T-cells [16]. Therefore, we have discussed 5-HT\textsubscript{3A} receptor in this paper.

Monoamine oxidase is a dimer protein of flavoprotein family responsible for the oxidation of monoamines such as serotonin. There are two isozymes of this enzyme: MAO-A and MAO-B [17]. MAO-A has affinity for serotonin and is found outside the central nervous system in the liver, gastrointestinal tract and placenta, while MAO-B is mainly present in blood platelets and has affinity for phenyl ethylamine. The genes of both enzymes are juxtaposed on x chromosome. Given the basic role of MAO in neutralizing the neurotransmitters, malfunction of this enzyme causes a variety of problems and diseases such as depression, schizophrenia, impaired concentration, migraine and abnormal sexual development [18]. Thus, this regulatory enzyme of serotonin level in peripheral blood, which is also a key enzyme of central nervous system, has been discussed in this article [19].

In this study, we evaluate the expression of 5HT\textsubscript{3A} receptor and MAO-A enzyme genes in mRNA level in PBMC of MS patients and healthy controls.

Materials and Methods

Study population

Subjects included 2 groups, the control group and MS patients, the
control group included 30 volunteers, they were healthy (19 Females and an 11 males, aged 22 to 45 years old). The target group consists of 30 MS patient. (19 Females and 11 males aged 22 to 45 years old) Sampling of Imam Khomeini Hospital of Tehran. All subjects consented to take part in this study and approved by ethical committee.

**PBMCs isolation and RNA extraction**

For each individual 5 ml of peripheral blood from cubital vein was obtained, and then transferred to Falcon 15 ml, containing 0.5 ml of ethylene di-amine tetra-acetic acid (EDTA). Samples transferred to laboratory in flask. Peripheral blood mononuclear cells isolated from 4 ml of whole blood each sample based on the gradient density centrifugation technique via Ficoll-hypaque [20]. Then the cells in phosphate buffered saline (PBS) were washed and cell viability was determined by trypan blue staining and concentration of cells was normalized (Concentration 7 × 10^6 cells/ml).the percentage of viable cells was more than 99%. Then, according to Roche high pure RNA isolation kit instructions (Roche, Germany), Total RNA was extracted. Finally, total RNA concentration of all samples normalized (70 ng/μl).

**cDNA synthesis, primer designing and PCR**

The synthesis of cDNA by using Primer Oligo (dT) and 11 ml mRNA and cDNA synthesis kit instructions (Fermentas, Germany) was performed. Then, final cDNA concentration and purity were detected by measuring its absorbance by means of NanoDrop 2000 instrument (Wilmington,USA) at 260/280 nm. Primer designing was performed for target genes and β-actin as internal control, for this purpose we used primer express software. Then, the designed primers were blasted (http://www.ncbi.nlm.nih.gov/tools/primerblast); they just adhered to accurate loci in mentioned gene (Table 1). PCR was performed by Using gene-specific primers and cDNA and according to manufacturer’s instructions PCR kit (Roche Germany). the product of PCR were loaded on 2% agarose gel and sharp appropriate bands were seen (Figures 1 and 2).

### Table 1: Primer used in quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Primer Sequence 5<code>-3</code></th>
<th>Length PCR (bp)</th>
<th>mRNA Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_001101</td>
<td>F-AGACCGAGGATGGCATTGGG\n R-GAGACGTTCACACGCCCCAGCC</td>
<td>161</td>
<td>B-actin</td>
</tr>
<tr>
<td>NM_001161772</td>
<td>F-GGTACCGGCCAGTACTGGACT\n R-CGGCGGATGACCACATAG</td>
<td>496</td>
<td>5-HT_R_A</td>
</tr>
<tr>
<td>NM_001270458</td>
<td>F-GCTGGACAAAGACTGCTAGGCGG\n R-GTTCTACCTTGGTCTCGAGGAGGT</td>
<td>180</td>
<td>MAO-A</td>
</tr>
</tbody>
</table>

**5-HT\_R\_A and MAO-A mRNA quantitation by real-time PCR**

Real-Time-PCR for target genes and β-actin was set up on Termo cycler Rotor-GeneTM6000 (Corbet Research, Australia) by using SYBR Green fluorescent dye (Light Cycler Fast Start DNA Master Plus SYBR Green 1, Roche, Germany). Rotor Gene instrument can detect the fluorescence emission radiation. We synchronized differences in total RNA volume of each reaction by using β-actin as internal control. Real-Time-PCRs were carried out on 1 μl cDNA templates by using 0.4 μl pairs of primers (200 nM β-actin, 5-HT\_R\_A and MAO-A) and 2 μl of SYBR Green 1 Master Mix in 10 μl of final reaction volume. appropriate RT-PCR programs were determined, so that conditions for β-actin were 10 s at 95°C, 10 s at 63°C and 10 s at 72°C, for 5-HT\_R\_A were 10 s at 95°C, 10 s at 62°C and 24 s at 72°C and for MAO-A were 10 s at 95°C, 10 s at 60°C and 12 s at 72°C. Finally, Real-Time PCR products were loaded on 2% agarose gel followed by staining the gel with Ethidium bromide.

**Statistical analysis**

First, we used LinReg PCR software for obtaining the Cycle of threshold (Ct) and efficiency of each reaction, and then data were imported to REST 2009 software, this software calculating is based on Pfaff equation which is as follow:

\[
P_{(H1)} = \frac{2^{-\Delta \Delta Ct}}{1 + 2^{-\Delta \Delta Ct}}
\]

In our study R shows Relative expression ratio of a target gene in comparison to control. (E) refers to real-time PCR efficiencies calculated by LinReg software, and (AC) imply the Ct difference between MS patients or exposed individuals against normal individuals (C\_normal-C, MS patients/exposed individuals).

**Results**

**Real-time PCR in MS patients and control group**

cDNA samples was gained from reverse transcription of mRNAs, then Real-Time PCR was performed on cDNA samples by using specific primers designed for 5-HT\_R\_A and MAO-A genes. SYBR Green fluorescent dye just can attach to double strand DNA and these fluorescence emission signals detected by Rotor gene at each cycle, this process followed by drawing a plot by instrument. If the first copy number of one sample is high, it is detected by Rotor gene earlier and vice versa. By using LinReg and REST2009 software and data analysis, we found that, relative expression of 5-HT\_R\_A receptor and MAO-A has shown significant increase (p<0.001) in PBMC of MS patients (Table 2 and Figures 1 and 2).

Sequencing of coding region of target genes (mutations in 5-HT3RA and MAO-A cDNA). The results of sequencing acknowledged accuracy sequence of 5-HT\_R\_A and MAO-A in PBMCs of attendants and MS patient no changes in their sequences.

**Discussion**

Multiple sclerosis (MS) is associated with lesions in brain and spinal cord that often lead to debilitating disease and sometimes death. MS is caused by autoimmune inflammatory invasion to myelin sheaths covering the neurons. T-cells in the thymus are sensitized against self-
The overexpression of this receptor in certain diseases, including the study of Ahangari et al. [18] in which the expression of this receptor was increased in patients with asthma and breast cancer. In addition, in a study conducted by Saberi et al. [20] overexpression of this receptor was indicated in lupus patients. In contrast, in a study in patients with schizophrenia and Tardive dyskinesia, it was found that the expression of this receptor in these diseases is reduced compared to healthy controls. These studies suggest that the expression pattern of this gene is different in various diseases and these changes may play a significant role in the development of these diseases.

van Heerden et al. in 2009 showed that the changes in expression of serotonin receptor genes of PBMC cells are similar to the changes occurring in neurons [34]. This is another indication of the impact of nervous system on disorders of immune system. Studies show that serotonin receptors affect lymphocytes as well as central nervous system [35]. According to the results of mentioned studies as well as what we know about Ca$^{2+}$-calmodulin-calcinuerin signaling pathway, influx of calcium into the cell exerts its effects through calcineurin, a calmodulin-Ca$^{2+}$ dependent protein phosphatase. This protein is activated and via dephosphorylation activates NFAT, a critical transcription factor transferred to the nucleus, which is important for a number of key genes involved in immune response of T-cells. Considering the presence of active calcium channels on T-cells, which open when ligand-gated potassium channels are opened as a result of calcium influx into the cell causing depolarization of the membrane [16], it can be presumed that this receptor activates the mentioned factor through influx of calcium into the cell, which ultimately activates T-cells. Therefore, the activation of T-cells is likely to be dependent upon the activation of type 3 serotonin receptors. According to our study, which is based on increased expression of type 3 serotonin receptor on PBMC of patients, perhaps increase in 5HT$_3$A receptor is a reason for development of MS symptoms. These symptoms include increased secretion of cytokines provoking increased proliferation and immunologic function of T-cells [16].

MAO is an enzyme of flavoprotein family. The gene of this enzyme is located on X chromosome and includes 91,917 base pairs in 16 exons [36]. This protein has two isozymes of A and B, with a higher affinity of the former for serotonin [17]. This enzyme not only degrades and neutralizes serotonin but epinephrine, norepinephrine, dopamine and other types of neurotransmitters. Thus, it is a key enzyme in the regulation of the activity of nervous system and any disruption in the function of this enzyme can cause severe neurological problems. Disorders such as depression, schizophrenia, impaired concentration and anti-social behavior have been attributed to malfunction of this enzyme [36].

There was significant change in the expression of MAO gene in MS patients compared to healthy subjects, which was increased in MS patients.

In the study of Bortolato and Shih [17], it was found that increased MAO-A level is associated with acute depression. Since depression is the first symptom of MS, MAO is likely to be a sign of depression in MS patients. The role of serotonin in MS is known from previous studies. Lower plasma level of serotonin is directly correlated with multiple sclerosis [17] and since MAO is the most important regulator of serotonin in the body [37], increasing expression of this enzyme can be justified in MS patients in whom impaired homeostasis of nervous system increases the expression of the gene encoding this enzymes, resulting in increased degradation of serotonin in plasma, which can be one reason for reduced serotonin level in this disease.

In this study, the impact of increased expression of 5HT$_3$A receptor...
on activation of T-cells as well as the role of MAO in progress of MS was examined for the first time. However, due to limitations of this study, we suggest further studies to ensure the exact mechanism of 5HT_A in T-cell function.

Conclusion

MS is developed due to autoimmune inflammatory attack to myelin sheath covering the neurons. With regard to different studies conducted on MS patients showing the involvement of T-cells in attacking myelin sheath, no study has been so far conducted on the role of serotonin receptor in the activation of T-cells nor the role of monoamine oxidase enzyme in progress of MS. According to the study of Hossein-Nezhad et al. [18] on the role of serotonin receptor in the activation of T-cells in autoimmune diseases like asthma and given the correlation between MAO enzyme with behavioral disorders, depression and migraine that can be a cause of depression in MS patients, we decided to study the role of serotonin receptor and MAO in this autoimmune disease. Although further studies are needed to determine the mechanism of disrupted expression of the gene encoding this receptor and enzyme in MS, we hope this study to be a step to find a definite safe therapy for multiple sclerosis.

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References