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Thioredoxin-1, redox factor-1 and thioredoxin-interacting protein, mRNAs are differentially expressed in Multiple Sclerosis patients exposed and non-exposed to interferon and immunosuppressive treatments

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ABSTRACT

Background: Oxidative stress is closely linked to inflammation in neurodegenerative diseases. We aimed to investigate the expression of redox system genes in Multiple Sclerosis (MS) patients either exposed or not exposed to conventional treatments.

Methods: Forty-four MS patients were divided into three groups: newly diagnosed (Group 1), receiving interferon (Group 2) and receiving immunosuppressive drugs (Group 3). Also, 15 healthy controls were enrolled. The mRNA expression of *TRX1*, *TXNRD1*, *TRX2*, *TXNRD2*, *TXNIP*, and *APEX1* genes in peripheral blood mononuclear cells (PBMCs) was assessed by relative quantitative real-time PCR. Also, serum level of Trx1 was measured by ELISA.

Results: Serum level of Trx1 in the newly diagnosed MS patients was significantly higher compared to the healthy controls ($P = 0.013$). Likewise, *TRX1* and *APEX1* expressions were significantly higher in the newly diagnosed patients compared to controls ($P = 0.003$ and $P = 0.042$), patients under interferon treatment ($P = 0.003$ and $P = 0.013$), and patients received immunosuppressants ($P = 0.001$ and $P = 0.025$). Furthermore, *TXNIP* expression in MS patients (either group 1, group 2, or group 3) was significantly lower than that in the control group ($P = 0.017$, $P = 0.002$, and $P = 0.022$ respectively). The expression of *TXNRD1*, *TRX2*, and *TXNRD2* did not show any significant difference between the control and the MS patient ($P > 0.05$).

Conclusions: Our data showed that redox system elements are differentially expressed in newly diagnosed MS patients, or patients receiving either interferon or immunosuppressive treatments. However, much more studies are required to confirm our findings and clarify the underlying mechanisms.

1. Introduction

Multiple Sclerosis (MS) is the most frequent chronic inflammatory demyelinating disease of the central nerve system (CNS) mainly affecting young people (Chaudhuri, 2013; Friese et al., 2014). The prevalence of MS disease has dramatically increased in recent years (Rezaali et al., 2013). It has been suggested that a combination of multiple factors such as genetic background, autoimmunity, oxidative stress, inflammation, vitamin D deficiency, viruses, etc., might be implicated in MS pathogenesis (Ciccarelli et al., 2014; Dendrou et al., 2015). Despite great attempts in this field, however, the actual cause and pathophysiological mechanisms of the disease remain unclear.

Oxidative stress is a biological cell damaging condition raised due to imbalance production and their neutralization of reactive oxygen species (ROS) by endogenous antioxidant systems (Wang et al., 2014). Oxidative stress has been closely linked to inflammation involved in cancers and neurodegenerative diseases (Ortiz et al., 2013; Trovato Salinaro et al., 2014; Vilhardt et al., 2017). There is a growing body of evidence on the role of oxidative stress, ROS-mediated cell damage and neuro-inflammation in initiation and progression of MS disease (van Horssen et al., 2011; Miljkovic and Spasojevic, 2013; Gironi et al., 2014; Guan et al., 2015; Ohl et al., 2016). Wang et al. (2014), for instances, remarked that oxidative stress following lipid peroxidation precedes the inflammatory response involved in demyelination and neuro-degeneration processes in MS patients. Also, Gironi et al. (2014) reported that oxidative stress differentially occurs in different courses of

Abbreviations: Trx1, Thioredoxin 1; Trx1R, Thioredoxin 1 reductase; Trx2, Thioredoxin 2; Trx2R, Thioredoxin 2 reductase; Txnip, Thioredoxin-interacting protein; Ref-1, Redox factor-1; MS, Multiple Sclerosis; RRMS, Relapsing-Remitting Multiple Sclerosis; SPMS, Secondary Progressive Multiple Sclerosis; CIS, Clinical Isolated Syndrome

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MS disease and might be a useful prognostic biomarker. Nonetheless, the redox system status -as a major endogenous counterpart of oxidative stress- and the reason for its failure to protect CNS from aforementioned detrimental effects of oxidative stress remain unclear in the context of MS disease.

The redox system mainly consists of thioredoxin (Trx), thioredoxin reductase (TrxR), NADPH and the thioredoxin-interacting protein (Txnip), a negative regulator of Trx (Mahmood et al., 2013). Trx molecule acts as an electron donor stabilizing unpaired-electron containing free radicals, and then the oxidized Trx is converted to its reduced form by the act of TrxR which uses NADPH as a source of electron (Holmgren and Lu, 2010). Under normal physiological conditions, there are two main Trx/TrxR circuits actively taking part in redox regulation: Trx1/Trx1R and Trx2/Trx2R. These circuits function in cytoplasm/nucleus and mitochondria, respectively (Lee et al., 2013). Also, a third circuit comprising of three testis-specific thioredoxin (Sptrx) and Trx GSH reductase has been found to be exclusively expressed in male germ cells (Lee et al., 2013). The Trx1/Trx1R is the most frequently studied circuits of the redox system in many diseases particularly cancers (Kakolyris et al., 2001; Welsh et al., 2002; Raffel et al., 2003; Penney and Roy, 2013). However, there is still a very short body of research about the of Trx1/Trx1R in pathogenesis of neurodegenerative diseases including MS (Pennisi et al., 2011; Mahmoudian et al., 2015) and Alzheimer's disease (Akterin et al., 2006). In addition, there are no evidences on the expression status of Trx2/Trx2R and other redox molecules in MS patients. Moreover, the impact of conventionally used MS treatments such as interferon and immunosuppressive drugs on the redox system elements is unclear.

In the present study, we aimed to assess the expression pattern of Trx1, Trx1R, Trx2, Trx2R, Txnip, and redox factor (Ref)-1 in newly diagnosed MS patients without any treatments as well as in established patients exposed to interferon or immunosuppressive treatments.

2. Materials and methods

2.1. Subjects

Forty-four MS patients including 38 women and 6 men, with a mean age of 31.8 ± 9.2 years old (range of 17–59 years old) were involved in the present study. Fifteen of these were newly diagnosed patients -within two weeks prior admitting to the study- who have not been taking any curative medications (MS Group 1). Twenty of the patients had been using beta-interferon drugs [eighteen patients on Interferon Beta-1A(30 μg i.m. once weekly) and two patients on Interferon Beta-1B (250 μg/ml subcutaneously every other day)] at the time of admission to the study which were assigned as MS Group 2. In beta Interferon group, only one patient received Oxybutynin in addition the main drug. Nine patients had been taking immunosuppressive treatments [eight patient on Azaram (50 mg TAB per day) and one patient on Betamethasone(4 mg/ml i.m. per 15 day)] who were assigned as MS Group 3. Also, 15 age and sex-matched healthy individuals including 11 women and 4 men with a mean age of 29.9 ± 10.4 years old (range of 19–59 years old) were enrolled as control group. The inclusion criteria for healthy control group included no history of autoimmune diseases, cancer, hypertension, or diabetes, and no history of taking beta-interferon, corticosteroid or immunosuppressive drugs within the 3 months prior to the study.

2.2. Ethics

The study was approved by the ethical committee located at the university. All the patients were requested to carefully read and sign an informed consent. No financial burden related to the study was imposed to the participants.

2.3. Sample preparation

A total of 10 ml blood sample was obtained from the antecubital vein from the all participants. From this, 5 ml was transferred into a tube containing ethylene di-amide tetra acetic acid (EDTA) anticoagulant (Merck, Darmstadt, Germany) for peripheral blood mononuclear cells (PBMCs) separation, and the remaining 5 ml was poured into a tube without any anticoagulant for serum separation. In order to separate buffy coat, the anticoagulant-containing blood samples were centrifuged at 2000 rpm for 10 min at 4 °C, and then approximately 600 μl of the creamy interphase layer of white blood cells was collected into 1.5 ml RNase- and DNase-free microtubes (Ratiolab GmbH, Dreieich, Germany), and preserved at − 20 °C until used for total RNA extraction. Also, the serum was prepared by centrifugation at 2500 rpm for 15 min at 4 °C, and stored at − 70 °C.

2.4. Real-time polymerase chain reaction (PCR) assay

Total RNA was extracted using RNXTM–PLUS kit (Sinaclon, Tehran, Iran) and turned into complementary DNA (cDNA) within 60 min at 45 °C using oligo dT primer Maxime RT-PCR PreMix kit (iNtRON Biotechnology, Gyeonggi, South Korea). The mRNA expression of *TRX1*, *TXNRD1*, *TRX2*, *TXNRD2*, *TXNIP*, and *APEX1* genes were then measured by relative quantitative real-time PCR using SYBR® Green Master Mix-Plus (Ampliqon, Odense, Denmark) as guided by the manufacturers, pairs of gene-specific primers and a real-time PCR thermocycler (Corbett RG6000, Australia). A housekeeping gene, β-*actin*, was used as internal control for normalization. Each reaction tube contained DNA polymerase mater mix (10 μl), mixed primers (F & R) (2 μl), and sample cDNA (2.5 μl). The net volume was reached to 20 μl by adding 5.5 μl Double distilled DNase free water. The primers we designed using GenScript Real-time PCR Primer Design online software (Available at; https://www.genscript.com/ssl-bin/app/primer). Also, some primers were selected as previously described (Javeri et al., 2013; Ogata et al., 2013). The oligonucleotide sequence of the primers has been given in Table 1. Each reaction was carried out in duplicates starting with an denaturation phase at 95 °C for 15 min, followed by 40 three-step cycles of denaturation at 95 °C for 10 s, annealing at 55–58 °C for 10 s and extension at 72 for 30 s. The reaction was terminated in a dissociation stage for recording the product melt (Higuchi et al., 1993). The cycling threshold (Ct) values of target genes (Ct_{target}) and beta-Actin ($Ct_{housekeeping}$) were used for calculation of gene expression by the following formulae: Gene expression $= 2^{-\Delta Ct} \times 1000$, where $\Delta Ct = Ct_{target} - Ct_{housekeeping}$.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The serum concentration of Trx1 was measured by ELISA method using commercial human Trx1 ELISA development kit (Product code: 3580-1H-6, Mabtech, Nacka Strand, Sweden), by following the manufacturer's guidelines. The level of sensitivity of the used kit was 20–2000 pg/ml. All measurements were performed in duplicates. The O-Phenylenediamine solution (Sigma-Aldrich Inc., USA) was used as chromogenic substrate for detection of Horseradish Peroxidase enzyme. After stopping the enzyme-substrate reaction with 50 μl of 1 M H_2SO_4, the color intensity in samples and standard wells was measured at 492 nm for test and 630 nm for reference using an Anthos 2020 microplate reader (Anthos, Wals, Austria). The Trx1 content of each sample was then calculated by extrapolation from the logarithmic formula curves generated in parallel with kit-provided standards.

2.6. Statistical analysis: statistical analysis

Data were analyzed using Prism Software version 6.04 for Windows (GraphPad, La Jolla, California, USA). Comparisons between the groups were examined by the Chi-square test for nominal values and by one-