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Evaluation of Rosuvastatin Therapy on *SIRT1* Gene Expression in Patients with Multiple Sclerosis: An Uncontrolled Clinical Trial

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ABSTRACT

Background: Multiple sclerosis (MS) is a chronic autoimmune disease. Current medications have some limitations such as low efficacy and high side effects. In recent years, statins have been raised as potential therapeutics for MS treatment with minimal complications. In addition, patient monitoring using suitable molecular markers is necessary for treatment response evaluation.
Objective: The aim of the present study was the evaluation of *SIRT1* gene expression changes following rosuvastatin therapy in patients with MS.
Methods: This before–after uncontrolled clinical trial study was performed on 25 patients with MS. Patients were treated with 20 mg rosuvastatin daily for 3 months. The Expanded Disability Status Scale (EDSS) was measured before and after statin therapy. Blood samples were taken from patients 2 times, before and after statin therapy, and centrifuged for white blood cell isolation. Total RNA was extracted using RNX-plus reagent, and complementary DNA was synthesized using Pars Tous cDNA Synthesis Kit. Real-time polymerase chain reaction was done using SYBR blue master mix and gene-specific primers in Roche light cycler. Patients' information was recorded using a checklist. Data analysis was performed using SPSS version 23 and Graph Pad version 9 software and $P < 0.05$ was considered a significant level.
Results: *SIRT1* was significantly upregulated in MS patients after statin therapy. Subsequently, EDSS of patients was decreased along with the increase in *SIRT1* gene expression, although EDSS changes were not significant ($P > 0.05$). Pearson correlation test showed no significant relationship between EDSS and *SIRT1* gene expression ($P > 0.05$). No significant relationship was observed between *SIRT1* expression or EDSS levels with patients' age, sex, weight, height, and body mass index and administrated drugs ($P > 0.05$).
Conclusions: *SIRT1* potentially is a sensitive and reliable biomarker for patients with MS monitoring during statin therapy.

Introduction

Multiple sclerosis (MS) is a central nervous system (CNS) disease characterized by chronic inflammation and immune response against myelin sheath.[1–3] The etiology of MS is unknown, but many environmental and genetic factors are involved in this disease.[4] Although the pathogenesis of MS is not completely determined, the main involved process includes the activation of peripheral T lymphocytes by antigen-presenting cells, migration of activated T lymphocytes through the blood-brain barrier, and spread of an immune reaction against the myelin sheaths, nerve axons, and oligodendrocytes. This process led to demyelination, axon loss, and nerve death due to excitotoxicity.[4] MS is the most common cause of nontraumatic disability that is more prevalent in women than men with an overall incidence of 2.5 million pa-

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tients worldwide.[5–7] This autoimmune disease can extremely influence quality of life and labor ability and impose a considerable psychological and economic burden on families and society.[8,9]

The main purpose of available treatments for MS is to prevent relapses or slow their progression because there is no cure for it yet.[4] These treatments have enormous limitations such as high cost, variable effectiveness (due to the complex pathology of the disease), considerable side effects, and inconvenient route of administration (injection).[7,10] Because more research is required for the discovery of novel and effective alternative medications with lower side effects.

Statins (eg, rosuvastatin, atorvastatin, and lovastatin) or hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors are oral medications used for hypercholesterolemia. HMG-CoA reductase is a rate-limiting enzyme in the de novo synthesis of cholesterol. Statins are tolerable with mild and uncommon side effects, including increased liver enzymes, rash, myalgia, peripheral neuropathy, and rhabdomyolysis (the last 2 complications are rare).[11,12] In previous studies, it has been shown that statins also have immunomodulatory and neurotrophic effects in addition to their cholesterol-lowering effects.[13,14] In vitro studies on immune cells isolated from patients with MS showed that statins have significant anti-inflammatory effects.[15–17] Animal model studies on experimental autoimmune encephalomyelitis (EAE) showed pleiotropic immune-modulatory and anti-inflammatory effects of statins.[18,19] Also, it has been shown that statins have neuroprotection and neurodegenerative effects and can enhance remyelination during EAE. They influence blood brain barrier integrity and prevent inflammatory cell infiltration into the CNS.[3,20–23] So, these medications have therapeutic potential in the treatment of MS as a CNS inflammatory disorder. However, human studies for evaluating statins' effects on MS treatment showed variable findings. Some studies showed a positive and enhancing effect[24–26] but some studies did not show considerable changes, whereas some patients showed negative effects on disease progression.[27]

Given the heterogeneous and unpredictable nature of MS, there is a problem in anticipating patients' prognosis or their treatment response. Therefore, there is a demand for the development of reliable biomarkers for the monitoring of disease progression and response to treatment.[28] Epigenetic modifiers, including histone deacetylases are involved in the expression regulation of immune, neuronal, and other tissue-specific genes.[29]

Sirtuin 1 (*SIRT1*) as a nicotinamide adenine dinucleotide-dependent histone deacetylases has an important role in regulating various biological processes, such as inflammation, homeostasis, oxidative stress, apoptosis, autophagy, and mitochondrial biogenesis.[1] There are many preclinical and clinical studies indicating the contribution of *SIRT1* in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, MS, inflammatory bowel disease, and so on.[30] In a study, *SIRT1* has been identified as a candidate gene related to pathological angiogenesis in autoimmune arthritis.[31] *SIRT1* also may be a potential biomarker of relapses, treatment response, and disease progression monitoring and also a potential therapeutic target in MS.[2]

In the present study, patients with MS were treated with rosuvastatin, and their disability level was determined using an Expanded Disability Status Scale (EDSS) before and after treatment. Also, the *SIRT1* expression level was measured using real-time polymerase chain reaction (RT-PCR) before and after treatment in all patients. Therefore, the first objective of the study is to investigate the effect of statin therapy on the improvement of patients with MS. Another aim of the study is to investigate the changes in *SIRT1* gene expression following statin therapy in patients with MS.

Materials and Methods

Participants and interventions

In this study, according to the results of previous studies, the SD and the maximum significant difference were considered equal to 0.015 and 0.01, respectively. Also, considering the limitations of the conducted studies, in this research, the sample size for independent societies was estimated, which is more than the sample size in paired matched societies. Using the following relationship, G*Power software (University of Dusseldorf, Dusseldorf, Germany) is available in the number of at least 27 samples according to the following formula. This uncontrolled (before–after) clinical trial study was performed on 25 patients with MS (2 patients were missed and did not complete the protocol) diagnosed by a neurologist. Study inclusion criteria were: magnetic resonance imaging findings consistent with the diagnosis, aged 20 to 70 years, LDL-C level <130 mg/dL, and patient satisfaction and cooperation. Exclusion criteria were taking drugs other than standard medicines, pregnancy, and breastfeeding. Withdrawal criteria were increase in patient disability and undesirable side effects. Also, patients with other inflammatory diseases were excluded from the study. All patients were treated with 20 mg rosuvastatin daily for 3 months. Patients' information (age, sex, height, weight, body mass index, and administrated drug) was recorded in a predesigned checklist.

Sample size calculation formula:

$$n = \frac{2(z_{1-\frac{\alpha}{2}} + z_{1-\beta})^2 \cdot \sigma^2}{d^2} = 27$$

$$\alpha = \%5, \beta = \%1, \sigma = 0.015, d = 0.01$$

Ethical considerations

This study was approved by the ethics committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.UMSHA.1400.503) and written informed consent was obtained from all patients. Required explanations were provided to the patients before the start of the study, and the patients' information was kept confidential. Furthermore, participation in this study was voluntary and there was no cost to the patients to participate in the study. Also, this clinical trial was recorded and approved by the Iranian Registry of Clinical Trials (IRCT20210211050323N2).

EDSS

EDSS is a scale for measuring disability levels in patients with MS. This scale scored from 0 (normal neurologic exam) to 10 (death from MS). This scale was measured 2 times in this study, the first time before the start of statin therapy and the second after the end of statin therapy. Clinical evaluation of patients was done based on EDSS.[32]

Sampling and RNA isolation

Blood samples (5 mL) were taken from patients 2 times, before and after statin therapy, and centrifuged for a buffy coat (mainly containing white blood cells) isolation. Total RNA was extracted using RNX+ solution (Cinnagen, Iran) according to manufacturer recommendations. Briefly, 1 mL RNX+ solution was added to 100 µL buffy coat and shaken for 30 seconds. After 5 minutes of incubation at room temperature, 200 µL chloroform was added to the mixture. Subsequently, samples were incubated at 4° C for 5 minutes. The samples were centrifuged at 12,000 rpm, 4° C for 15 minutes, and the aqueous phase was transferred to a new tube. The same volume of ice-cold isopropanol was added to the tube, gently mixed, and kept at –20 C° for 30 minutes. Again, samples

Table 1
The gene-specific primer pairs used in real-time polymerase chain reaction.

Gene symbol	Primer sequence (5÷3)
SIRT1	Forward: CTTCACCACCAGATTCTTCAG
	Reverse: TTCAGCAATACTTTCAACATTCC
GAPDH	Forward: AAGGCTGTGGGCAAGGTCATC
	Reverse: GCGTCAAAGGTGGAGGAGTGG

were centrifuged at 12,000 rpm, 4° C for 15 minutes, and the supernatant was discarded. Next, 1 mL 75% ethanol was added to the RNA pellet and was vortexed to dislodge the pellet. The supernatant was discarded and 50 µL sterile nuclease-free water was added to the pellet after partial drying at room temperature. DNase I (Sigma Aldrich, St Louis, Missouri) was used for the elimination of any potential genomic DNA contamination in RNA samples. RNA concentration and purity were determined by reading its absorbance in 260 nm and 280 nm wavelengths using a nanodrop spectrophotometer. RNA integrity was confirmed using electrophoresis on 1.5% agarose gel.[33]

cDNA synthesis and RT-PCR

Complementary DNA was synthesized by First Strand cDNA Synthesis Kit (Pars Tous, Iran) using 1 µg total RNA according to manufacturer guidelines in a final volume of 20 µL. Gene expression was measured quantitatively and was performed using SinaSYBR Blue HS-qPCR Mix (Sinaclone, Iran) by Roche Light Cycler 96 RT-PCR instrument according to kit instructions. The sequence of forward and reverse gene-specific primer pairs are shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase was considered a housekeeping gene. Briefly, 10 µL SinaSYBR Blue, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 2 µL complementary DNA, and 4 µL nuclease-free water were added to a tube for each reaction. After 5 minutes of preincubation of samples at 95 °C, an RT-PCR was performed in 45 cycles (denaturation: 95 °C for 5 minutes, annealing: 59 °C to 61 °C for 30 seconds, extension: 72 °C for 30 seconds). After 3-step amplification, melting analysis was done to confirm product specificity. Product specificity also was confirmed by electrophoresis of RT-PCR products on 1% agarose gel. Reaction efficiency was calculated using the $E = +10^{(-1/slope)} - 1$ formula. Expression fold change was calculated using the $F = 2^{-\Delta\Delta CT}$ formula.[33,34]

Statistical Analysis

Data are shown as mean (SD) or frequency (%). Data were analyzed using paired sample *t* test, independent samples *t* test, 1-way analysis of variance, and Pearson correlation coefficient by SPSS version 23 (IBM-SPSS Inc, Armonk, New York) and Graph Pad Prism version 9 (La Jolla, California). $P < 0.05$ was considered a significant level.

Results

Patient descriptions

The participants (n = 25) included 6 men (24%) and 19 (76%) women. Patients are described in Table 2.

SIRT1 *expression changes after statin therapy*

Quantitative gene expression analysis showed that *SIRT1* significantly upregulated in patients with MS after 3 months of statin therapy ($P < 0.001$) (see panel A of the Figure). However, the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, the expression did not show any significant change following statin therapy ($P > 0.05$).

Table 2
Description of study participants.

Variable	Frequency
Sex	
Male*	6 (24)
Female*	19 (76)
Age (y)†	44.52 (10.97)
Weight (kg)†	65.96 (11.71)
Height (cm)†	165.44 (7.38)
Body mass index†	24.18 (4.44)
Administrated drugs*	
Dalfyra	2 (8)
Doxorubicin	1 (4)
Fingolimod	7 (28)
Rituximab	12 (48)
CinnoVex	2 (8)
Others	1 (4)

Dalfyra® (Nano Hayat Darou Company): Fampridine CinnoVex® (CinnaGen Company): interferon beta-1a.
* Values are presented as n (%).
† Values are presented as mean (SD).

Table 3
Relationship between Sirtuin 1 (SIRT1) gene expression with patients Expanded Disability Status Scale (EDSS), age, weight, height, and body mass index (BMI).

Variable	Sirtuin 1 gene expression*	
	Before R. T†	After R. T
EDSS Before R. T	‡$r = -0.091$	$r = -0.109$
	§P value = 0.678	P value = 0.621
EDSS After R. T	$r = 0.304$	$r = 0.218$
	P value = 0.159	P value = 0.319
Age	$r = -0.120$	$r = 0.086$
	P value = 0.587	P value = 0.697
Weight	$r = -0.064$	$r = 0.386$
	P value = 0.772	P value = 0.069
Height	$r = -0.187$	$r = -0.053$
	P value = 0.394	P value = 0.811
BMI	$r = 0.107$	$r = 0.396$
	P value = 0.626	P value = 0.060

* XXXXX.
† R.T: Rosuvastatin Therapy.
‡ r: Regression.
§ p-value: Significance considered as < 0.05.

EDSS Relationship with SIRT1 *expression*

EDSS of patients was decreased along with the increase in *SIRT1* gene expression, although EDSS changes were not significant ($P > 0.05$) (see panel B of the Figure). Also, the Pearson correlation test showed no significant relationship between EDSS (before or after statin therapy) and *SIRT1* gene expression (before or after statin therapy) ($P > 0.05$) (Table 3).

Relationship between patients' demographic data and SIRT1 *expression and EDSS levels*

No significant correlation was observed between *SIRT1* expression (Table 3) and EDSS levels (Table 4) with age, weight, height, and body mass index. Also, there was no significant relationship between patients' sex and administrated drugs with *SIRT1* expression or EDSS levels (Table 5).

Discussion

Currently, there is no curative treatment for MS, an autoimmune disease,[4,6] and the purpose of the available treatments is to slow down the MS progression and prevention of disease relapse.[4] As far as we know, no study has investigated the changes in *SIRT1* expression following statin therapy in patients with MS and this is the strength of the present study.

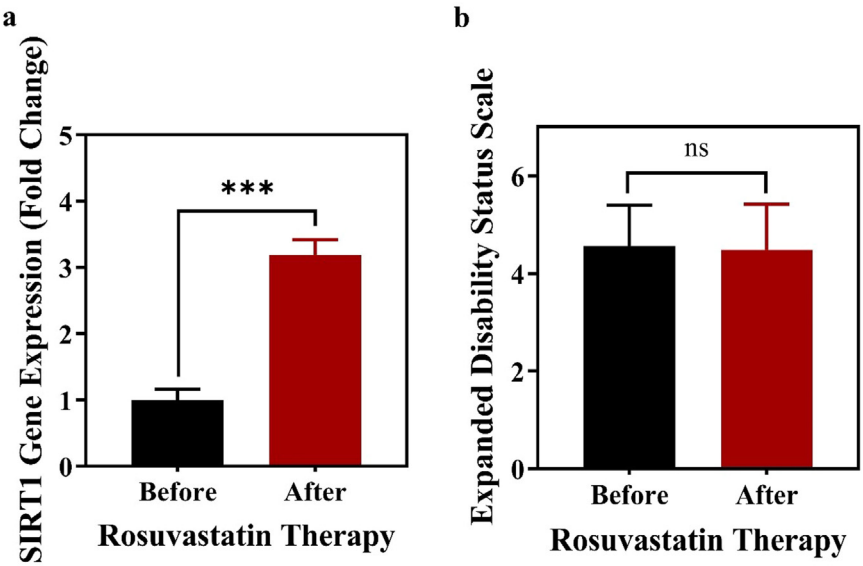

Figure. SIRT1 expression and Expanded Disability Status Scale (EDSS) changes following statin therapy. A) Comparison of *SIRT1* gene expression before and after treatment with rosuvastatin. B) Comparison of EDSS level before and after treatment with rosuvastatin. ns = not significant. ***Significant at $P < 0.0001$.

Table 4
Relationship between patients Expanded Disability Status Scale (EDSS) levels with age, weight, height, and body mass index (BMI).

Variable	EDSS	
	Before R. T*	After R. T
Age	†$r = 0.120$	$r = 0.047$
	‡P value = 0.568	P value = 0.824
Weight	$r = 0.039$	$r = 0.007$
	P value = 0.855	P value = 0.973
Height	$r = -0.016$	$r = -0.071$
	P value = 0.938	P value = 0.735
BMI	$r = 0.063$	$r = 0.047$
	P value = 0.764	P value = 0.824

* R.T: Rosuvastatin Therapy.
† r: Regression.
‡ p-value: Significance considered as < 0.05.

In the present study, 25 patients with MS were given rosuvastatin for 3 months. *SIRT1* expression in peripheral blood white blood cells was evaluated before and after therapy using RT-PCR. Also, the patient's EDSS level was determined to clinically assess the patient's disability. The results showed that rosuvastatin therapy significantly enhanced *SIRT1* expression.[35] A decrease in *SIRT1* expression is more common in other diseases such as MS, cardiovascular disease, Parkinson disease, and Alzheimer disease.[36,37–41] This means that a decrease in *SIRT1* gene expression is associated with further progression of the disease as an elevation in inflammation and oxidative stress.[40] Similar to our study, previous studies demonstrated that *SIRT1* expression is decreased in patients with MS during the relapse phase and also in nonresponders to glatiramer acetate treatment.[28,42] The elevated levels of *SIRT1* expression appear to be associated with increased brain-derived neurotropic factor and Nicotinamide adenine dinucleotide levels, which provide protective phenotype.[43] *SIRT1* exerts cell survival and its antiapoptotic, anti-inflammatory, and immunomodulatory effects through NFkB, p53, P300, NOs, FOXO, PGC1, Akt, STATs, and pathways.[28]

Previous studies indicated that statins exert their neuroprotective and immunomodulatory functions in CNS autoimmune diseases by several mechanisms.[44,45] Statins inhibit HMG-CoA reductase that can control the geranylgeranyl-pyrophosphate and farnesyl-pyrophosphate formation in addition to cholesterol de

Table 5
Relationship between patients' SIRT1 expression and EDSS with sex and administrated drugs.

Variable	EDSS (SD)		Sirtuin 1 gene expression† (SD)	
	Before R. T*	After R. T	Before R. T	After R. T
Sex				
Male	4.16 (0.81)	4.08 (0.97)	−0.42 (3.08)	2.48 (3.48)
Female	4.68 (0.83)	4.60 (0.92)	0.87 (4.27)	4.03 (4.03)
P value‡	0.197	0.244	0.505	0.414
Administrated drugs				
Dalfyra	4.25 (0.35)	4.25 (0.35)	−2.88 (4.21)	4.40 (6.61)
Doxorubicin	3.5 (0.00)	3.00 (0.00)	−2.72 (0.00)	2.74 (0.00)
Fingolimod	4.57 (0.83)	4.85 (1.18)	1.81 (5.90)	5.86 (5.28)
Rituximab	4.66 (1.00)	4.41 (0.92)	0.30 (2.85)	2.38 (3.14)
CinnoVex	4.75 (0.35)	4.50 (0.00)	2.46 (4.65)	3.41 (0.89)
Others	4.50 (0.00)	4.50 (0.00)	1.57 (0.00)	3.66 (0.00)
P value	0.862	0.624	0.695	0.709

EDSS = expanded disability status scale; R.T: Rosuvastatin Therapy; r: Regression; P value: Significance.
* R.T: Rosuvastatin Therapy.
† SD: standard deviation.
‡ p-value: Significance considered as < 0.05.

novo synthesis. These factors are 2 main parts of isoprenylation and, as a result, the function of guanosine triphosphate-ases such as Rho, Ras, Rab, and Rac proteins that are involved in cell migration, differentiation, proliferation, and so on.[46] Another possible mechanism is cholesterol-dependent, in such a way that depletion of cholesterol resulting from the inhibitory effect of statins on HMG-CoA reductase may influence the assembly of lipid rafts in the cell membrane. Lipid rafts act as a platform to concentrate and anchor receptors and other proteins contributing to signal transduction to interaction with ligands and other molecules.[47] A significant increase in *SIRT1* expression was reported in a study by Yamac et al[48] following statin therapy and a potential cardioprotective role in myocardial infarction was suggested. Another study by Ota et al[49] showed that statin therapy enhances *SIRT1* expression levels and prevents endothelial senescence through a direct effect on the Akt pathway.[49] Paul et al[26] indicated that high-dose atorvastatin treatment in patients with MS is safe and well tolerated and magnetic resonance imaging findings show a beneficial effect as a result of the immunomodulatory effect of atorvastatin. Sun et al showed that combined treatment with Nicotinamide adenine dinucleotide and atorvastatin could slow down the progression of EAE by immune regulation through several pathways such as *SIRT1* expression induction. Other studies reported that statin-based treatments protect the peroxisomal/mitochondrial axis and therefore exert efficient neuro repair and neuroprotection effects under EAE/MS conditions, in addition to their immunomodulatory and anti-inflammatory properties.[49–51] However, in the present study, *SIRT1* elevation was not significantly associated with EDSS improvement. Evidence suggests that the EDSS has low sensitivity and may not be able to measure small changes in the level of disability.[52] On the other hand, changes at the molecular level may occur earlier than changes at the cellular and multicellular levels.[53] The main limitation of this study was the unavailability of a control group and the small sample size

Conclusions

In the present study, *SIRT1* gene expression was significantly induced following 3 months of statin therapy (rosuvastatin). However, EDSS reduction as a late response probably needs more time to produce significant changes. Therefore, *SIRT1* potentially is a sensitive and reliable biomarker for early evaluation of MS treatment response.

Declaration of Competing Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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