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Original Article

Regulatory Effects of Various IFN-β Formulations Therapy on CXC Chemokines CXCL1 and CXCL9 Gene/Protein Levels in Relapsing-Remitting Multiple Sclerosis Patients

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Abstract

Multiple sclerosis (MS) is a complex clinical immune system disorder. The most common symptoms of MS are recurrent loss of myelin in conjunction with inflammation in the central nervous system (CNS). Chemokines, as important immune system components, play a role in immune responses. This project aimed to examine and compare the serum levels of CXCL1 and CXCL9 in patients with relapse-remitting MS (RRMS) following therapy with IFN-β formulations. Clinical specimens were collected from 50 unrelated healthy controls, as well as 40 RRMS patients treated with Cinnovex™ (CVX, Interferon beta-1a, made in Iran) and Avonex® (AVX, Interferon beta-1a, made in the USA). The fold changes in gene expression of CXCL1 and CXCL9 compared to the β-actin gene were determined using real-time PCR. The protein expression and serum levels of CXCL1 and CXCL9 were measured using the ELISA method. Data analysis was performed using the ANOVA test. The levels of CXCL1 and CXCL9 significantly increased, with greater upregulation observed with AVX and, to a lesser extent, with CVX. Our study results suggest that AVX is more effective than CVX in regulating the immune system, and the dosage of CVX may need to be increased to achieve a more pronounced therapeutic response in RRMS patients.

Keywords: Avonex®, Cinnovex™, CXCL1, CXCL9, IFN-β Formulations, Multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is a complex immune system disorder characterized by the recurring loss of myelin in the central nervous system (CNS) [1, 2]. The specific mechanism of demyelination in MS is not fully understood [3], but scientific research indicates that both genetic and environmental factors contribute to the development and complications of the disease [4, 5]. Recent studies have focused on the role of immune system molecules like chemokines and cytokines in understanding the causes and progression of MS [6, 7]. To this

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point, the four most important businesses of chemokines have been identified based totally on the wide variety of function of cysteine residues of their biochemical structure: CXC, CX3C, C, and CC [8, 9]. Over fifty chemokines and twenty chemokine receptors have been diagnosed. Multiple members of the CXC chemokines have been showed to be concerned in various inflammatory conditions, consisting of diabetes [10], multiple sclerosis [11], food allergies [12)], pre-eclampsia [13], osteoporosis(14)], and HBV(15)]. In Iran, there are two commercially available interferon-β formulas for treating patients with relapsing-remitting MS (RRMS): Cinnovex™ (CVX, Cinnagen Inc, Tehran, Iran) and Avonex® (AVX, Biogen Inc, MA, USA). These medications are approved as the first line of therapy in the Iranian market by the Iranian Ministry of Health and Medical Education [16]. Previous studies have used Real-Time PCR analysis to demonstrate that the CCL2 subfamily is significantly elevated in RRMS patients after B-interferon therapy [17]. Additionally, elevated levels of other chemokines, such as CXCL12, have been reported in both active and inactive MS lesions [6, 18]. Therefore, any interferon-β formula that regulates CXCL1 and CXCL9 could be a promising treatment option for RRMS patients. It has been observed that there is an increased percentage of peripheral blood T cells expressing the chemokine receptors CXCR3 and CCR5 in MS patients, which correlates with disease activity. These findings provide evidence for the involvement of CXCR3 and CCR5 in the pathogenesis of MS [6, 19]. Our previous study found that CVX has less ability to downregulate levels of cytokines like IL-17 and IL-12 and upregulated levels of the cytokine IL-10 compared to other IFNs [20]. A recent study by Hao and colleagues also reported that CXCL9 levels remained unchanged after IFN-β therapy in MS patients [21]. In a separate study, La Rosa et al. [22] pronounced that IFN-β may additionally lower inflammation via lowering chemokine expression in PBMCs at the blood-mind barrier (BBB) inside MS lesions [20]. Usually, IFN-β is produced using maximum cellular sorts in reaction to viral infections. It activates various genes, inhibits virus replication, slows the increase of goal cells to cause them to be more vulnerable to apoptosis, and has huge immunomodulatory outcomes, making it an effective inducer of antiviral protection [23]. Most chemokine promoters contain elements that IFN-β can induce, and the molecular mechanism for the inducibility of some of these promoters has been explained by IFN-β [24]. Based on this background, our study aims to investigate the levels of CXCL1 and CXCL9 in the serum of Iranian patients with relapse-remitting multiple sclerosis (RRMS) who have received different IFN-β formulations. The objective of this project is to examine and compare the levels of CXCL1 and CXCL9 in the serum of Iranian RRMS patients after treatment with various IFN-β formulations.

2. Materials and Methods

2.1. Patients and specimens

Clinical specimens were obtained from 50 unrelated healthy controls and 40 RRMS patients treated with Cinnovex™ (CVX, Interferon beta-1a, made in Iran) and Avonex® (AVX, Interferon beta-1a, made in the USA). Specimens were taken at the Special Diseases Department in Ali Ibn Rafsanjan Hospital,

Rafsanjan, Iran. One group of patients received intramuscular injections of 30µg/week CVX. The second group of patients received intramuscularly 30µg/week of AVX. An expert neurologist confirmed the development of MS concerning both clinical and paraclinical evidence (MRI study, oligoclonal bands in cerebrospinal fluid, and evoked potentials) according to McDonald's criteria [25]. Members of the RRMS group had the same percentage of sex, age, disease duration, and socioeconomic position. Evaluation of socioeconomic standing was obtained with a questionnaire based on the levels of education (including foundation course: low; undergraduate studies: moderate; and postgraduate) and monthly income (below $250.00: low; $250.00-1000.00: moderate; and more than $1000.00: high).

All healthy controls were chosen from the Rafsanjan population with a similar ethnic group, sex, age, and socioeconomic position. The ethics committee of Rafsanjan University of Medical Sciences accepted the project protocol before performing, and the patients and control groups filled out written consent forms before sample collection.

2.2. Measurement of chemokine serum levels

The effects have been expressed as pg/mL. Serum CXCL5 levels were tested using an ELISA package (R&D structures, United Kingdom) following the manufacturer's instructions.

2.3. RNA extraction and generation of cDNA and Real-time PCR

Using the Parstoos TM Whole RNA Prep package, whole RNA was recovered from tissue to detect the expression of cytokines, and NanoDrop was used to measure RNA attention. 1 µg of total RNA and the cDNA opposite transcription package (Parstoos) were used to synthesize cDNA. NanoDrop takes care of the purity of the obtained cDNA. β-actin was used as a house task gene for this investigation, and SYBR® green PCR master mix (Biosistem) was utilized to detect the expression of genes in real-time.

SPSS 18 software was used for the statistical analysis, with a significance threshold of $p<0.05$. Disparities between the groups were assessed using the $\chi 2$, t-test, and ANOVA with a 90% test power in **Table 1**.

2.4. Statistical analysis

Statistical analysis was performed using SPSS 18 software, and the significance level was set at $p<0.05$. differences between groups were determined by $\chi 2$, t-test, and ANOVA employing a power of the test of 90%.

Table 1. Indicates the Sequences of the Employed Primers in this Study.

Target	Primer	amplicons lengths	length of primer	Tm
CXCL1	F: 5′-CAAACCGAAGtCATAGCCACA-3′ R: 5′-CTCCTAAGCGATgCTCAAACA-3′	250 bp	21 21	58.58 58.38
CXCL9	F: 5′-GTGGTGTTCTTTTCCTCTTGG-3′ R: 5′-ATAGTCCCTTGGTTGGTGCT-3′	112 bp	21 20	57.01 58.63
β-actin	F: 5′-GGGCATGGGTCAGAAGGATT-3′ R: 5′-CGCAGCTCATTGTAGAAGGT-3′	150 bp	20 20	59.74 57.98

3. Results and Discussion

The patients' and control groups' mean ± SD ages were 40±9 and 40±7 years, respectively (p=0.85). Of the patients, 41 (59%) were female and 29 (41%) were male; in the control group, the distribution was 60 (60%) female and 40 (40%) male (p=0.9). Regarding socioeconomic position, there was likewise no discernible difference between the two groups (p=0.90). Each of the two groups was paired.

3.1. Results of measurement of chemokine serum levels

CXCL1 significantly increased in the presence of both formulations in patients compared to the control. The CXCL1 levels were 288 ± 3.2 pg/ml and 228 ± 3.8 pg/ml when patients were treated with AVX and CVX, respectively. The CXCL1 serum levels in healthy controls were 106 ± 0.9 pg/ml. CXCL1 levels in different IFN formulations indicated a significant difference compared to healthy controls. There was also a significant difference in the level of CXCL1 when RRMS was treated with CVX compared to AVX (P<0.05) (**fig. 1**).

Results of our study also showed that CXCL9 significantly increased in the presence of all formulations compared to the control. It was 449.2 ± 2.2 pg/mL and 228.1 ± 3.8 pg/mL when patients were treated with AVX and CVX, respectively. The CXCL9 levels in healthy controls were 109.7 ± 1.01 pg/ml. CXCL9 levels were significantly changed in patients treated with different IFN formulations compared to healthy controls. There was also a significant difference in CXCL9 levels when RRMS was treated with CVX compared to AVX (P<0.05) (**fig. 2**).

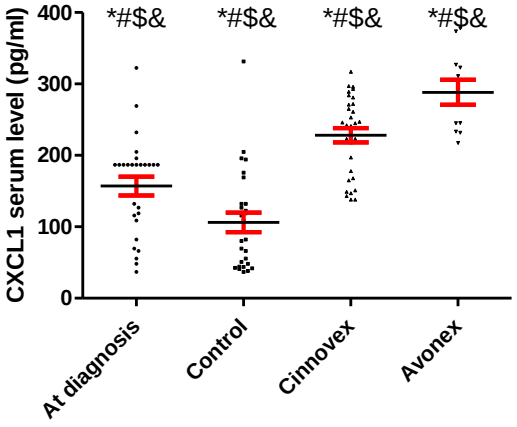

Figure 1. Demonstrate Serum Protein Levels of CXCL1 Before (at Diagnosis) and Following Treatment with Cinnovex and Avonex in comparison to Control

* significant in comparison with diagnosis and three other groups (Control, Cinnovex, and Avonex) (P<0.05).
# significant in comparison with Control and three other groups (At diagnosis, Cinnovex, and Avonex) (P<0.05).
$ significant in comparison with Cinnovex and three other groups (Control, At diagnosis, and Avonex) (P<0.05).
& significant in comparison with Avonex and three other groups (Control, Cinnovex, and at diagnosis) (P<0.05).

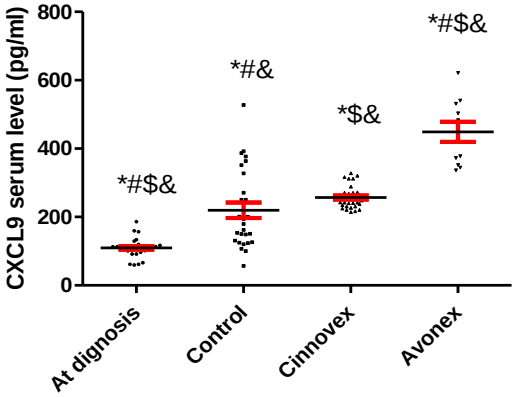

Figure 2. Demonstrate Serum Protein levels of CXCL9 Before (at Diagnosis) and Following Treatment with Cinnovex and Avonex in Compare to Control

* significant in comparison with At diagnosis and three other groups (Control, Cinnovex, and Avonex) (P<0.05).
# significant in comparison with Control and two other groups (At diagnosis and Avonex) (P<0.05).
$ significant in comparison with Cinnovex and two other groups (At diagnosis and Avonex) (P<0.05).
& significant in comparison with Avonex and three other groups (Control, Cinnovex, and At diagnosis) (P<0.05).

3.2. Results of Real-time PCR:

Our results confirmed that the mRNA expression of CXCL1 was significantly upregulated before (at diagnosis) and following treatment with Cinnovex and Avonex compared to the control (P<0.05) (**fig. 3**).

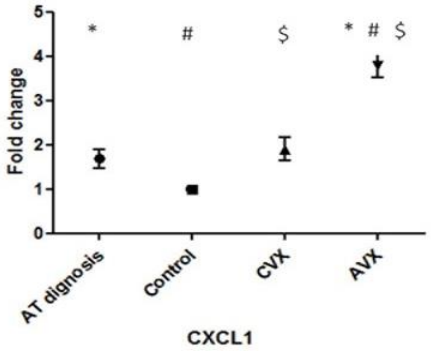

Figure 3. Demonstrate Expression of the CXCL1 Gene Before (at Diagnosis) and Following Treatment with Cinnovex and Avonex compared to Control.

* significant in comparison with At diagnosis and three other groups (Control, Cinnovex, and Avonex) (P<0.05).
# significant in comparison with Control and three other groups (At diagnosis, Cinnovex and Avonex) (P<0.05).
$ significant in comparison with Cinnovex and three other groups (Control, At diagnosis, and Avonex) (P<0.05).
& significant in comparison with Avonex and three other groups (Control, Cinnovex, and at diagnosis) (P<0.05).

Our results confirmed that the mRNA expression of CXCL9 was significantly upregulated before (at diagnosis) and following treatment with Cinnovex and Avonex compared to the control (P<0.05) (**fig. 4).**

In order to minimize potential confounding factors, we carefully matched the RRMS patients and controls in our study based on their demographic and socioeconomic characteristics. Previous studies have demonstrated that treatment with IFN-β in vivo can increase the expression of CXC chemokines, making it a key regulator of chemokine expression. Our findings are in line with the results study by Metzemaekers et al. (2018) that CXC chemokines CXCL9, CXCL10, and CXCL11 are predominantly induced by interferon (IFN)-γ [26]. Reports indicate that macrophages express CXCL10 and CXCL9 proteins within actively demyelinating lesions and by reactive astrocytes in the surrounding parenchyma. CXCR3 is expressed via T cells and astrocytes in the lesion. It has been revealed that during the inflammatory response, IFN-γ stimulates glial cells to produce CXCL10 and CXCL9, which selectively recruit activated T-lymphocytes into the CNS [27]. Therefore, these cell types may produce some of the serum chemokines in our study. Similarly, Smolders et al. (2020) reported that CSF levels of CXCL9 and CXCL10, which act on T cells and mononuclear phagocytes, are well documented during MS attacks [28].

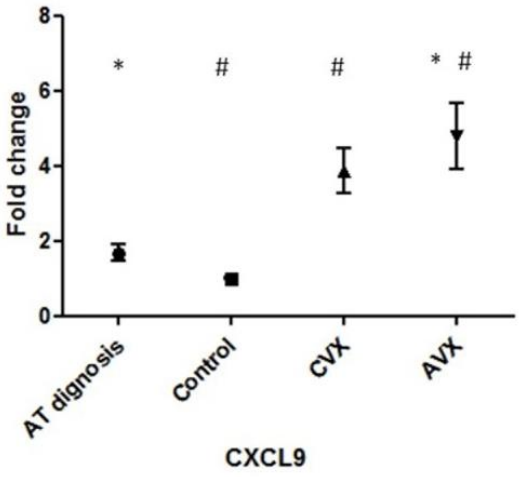

Figure 4. Demonstrate Expression of CXCL9 Gene Before (at Diagnosis) and Following Treatment with Cinnovex and Avonex in Compare to Control.

* Significant difference between At diagnosis and three other groups (Control, Cinnovex, and Avonex) (P<0.05).
# Significant difference between Control and three other groups (At diagnosis, Cinnovex and Avonex) (P<0.05).
$ Significant difference between Cinnovex and three other groups (Control, At diagnosis, and Avonex) (P<0.05).
& Significant difference between Avonex and three other groups (Control, Cinnovex, and at diagnosis) (P<0.05).

Investigations into the expression of chemokine receptors in RRMS patients treated with IFN-β have shown increased CCR4 expression on CD4+ T cells [6], similar to our findings. This suggests a potential therapeutic role for chemokines in RRMS patients. In another study, Buttmann et al. (2004) demonstrated that IFN-β therapy leads to a significant but temporary increase in plasma levels of CXCL10, another member of the same subfamily, in patients with stable RRMS [29]. Patients who obtained I.M. Injections once a week showed a more potent systemic chemokine response than those treated with S.C. injections of Betaferon and Rebif twice weekly, similar to our AVX-treated patients. Additionally, AVX has been shown to induce chemokines regardless of when therapy is initiated, which is consistent with our results [29]. Compelling evidence suggests that the expression pattern of cytokines/chemokines plays a role in the development of MS [20, 30]. Interestingly, previous research has shown that IFN-b formulations utilized in RRMS remedy down-regulated stages of inflammatory cytokines and up-regulated degrees of anti-inflammatory cytokines in these patients. Previous studies have shown that in those patients, IFN-b formulations utilized in RRMS treatments lessen degrees of inflammatory cytokines and boom degrees of anti inflammatory cytokines [20, 31, 32]. It is well-documented that MS patients experience enhanced pro-inflammatory immune responses and autoimmunity, including IFN-g, TNF-α, and IL-17 [33, 34]. Higher levels of pro-inflammatory cytokines have also been found in untreated MS patients [31, 35]. CXCL1 and CXCL9 are involved in inflammatory responses and belong to the inducible chemokine family. Our previous research has shown that both CXCL10 and CXCL12 significantly increase in RRMS patients, regardless of their known genetic variations. Additionally, we have demonstrated that pro-inflammatory cytokines like TNF-α and INF-γ in other cell systems induce these chemokines. Therefore, it can be assumed that elevated inflammatory cytokines may be a possible mechanism for the upregulation of these chemokines in RRMS patients. In conclusion, there is a direct relationship between cytokine expression and chemokine expression [20]. Sorensen et al. in 2002 and Zhou et al. in 2019, in two separate studies, have reported that CXCR3 (the receptor for CXCL9) is selectively suppressed on CD3+/CD8+/CXCR3+ expressing cells in MS patients after three months of β-interferon therapy [36, 37]. It can be speculated that the mechanisms of action of IFN-b formulations in controlling inflammation involve the modulation of chemokine/chemokine receptor expression. The corresponding CXC chemokine receptors for CXCL1 (CXCR2) and CXCL9 are expressed by oligodendrocytes and astrocytes, and their chemokine ligands are expressed in vitro in response to pro-inflammatory cytokines IL-1β and IFN-γ [40]. Together with our findings, the increased chemokine level may contribute to demyelination by inducing oligodendrocyte recruitment to the MS lesions. However, based on our knowledge, this study is the first to investigate the regulatory effect of IFN-b therapy on chemokine expression. Evidence suggests that other members of the CXC

chemokine family, such as CXCL8, and the member of the CC family, CCL2, are reduced following IFN-β1α (AVX) therapy in RRMS patients [38, 39]. Elevated serum levels of CXCL1 and CXCL9 in untreated patients compared to the control group indicate that CVX modulates the immune system less effectively than AVX in our studied MS patients. In our results, circulating levels of both CXCL1 and CXCL9 were upregulated following treatment with CVX in RRMS patients. However, Krakauer et al. reported that CXCL9 remained unchanged following INF-β therapy in MS patients [40]. IFN-β may also exert anti-inflammatory movement via decreased PBMC chemokine expression on the BBB within MS lesions [23]. Primarily based on the latest results, we speculate that IFN-β injection might result in transient systemic chemokine bursts in MS patients. Accelerated chances of peripheral blood T cells expressing the CXCR3 and CCR5 chemokine receptors were said in sufferers with MS, correlating with ailment activity. Those findings assist a function for CXCR3 and CCR5 in MS pathogenesis [44].

Our previous study also showed that CVX has less power to down-regulate inflammatory cytokines, such as IL-12 and IL-17, and upregulate the anti-inflammatory cytokine (IL-10) than other IFN formulations [20]. Therefore, it seems that CVX is a weaker component than other IFN-b formulations in down-regulating inflammatory chemokines. Some plausible reasons that may have affected our investigation and studied patients could be summarized as concluding remarks of the study:

a) RRMS patients treated with CVX were more numerous than those who received other compounds, which may have provided greater statistical power in identifying significant changes in serum levels of chemokines than AVX.

b) Patients received lower doses of CVX than AVX, which may have affected the results. It is suggested that more CVX is needed to down-regulate immune system functions and overcome disease complications.

c) CVX is a homolog of AVX. This newly synthesized compound requires more studies to investigate its efficacy, side effects, and mechanisms of action on the immune and other body systems. Further studies are needed to accurately address the effects of β-INF formulations, including CVX, on immune system components, including chemokines and cytokines, both at the mRNA and protein levels, to verify the mechanisms involved in their impacts on immune responses in MS.

4. Conclusion

The results of our study suggest that AVX is much more powerful than CVX in regulating the immune system, and the dose of CVX probably should be increased to achieve an overt therapeutic response in RRMS patients. Further studies and follow-up with these drugs are beneficial in discovering all aspects and achieving a definitive outcome.

Conflict of interest

The authors declare to have no conflict of interest.

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