Short report of potential Myelinogenesis effects of taper up-off of opium tincture in rodent model of multiple sclerosis

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Short Report

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Abstract

Multiple sclerosis (MS) is one of the most common demyelinating autoimmune diseases that affects the central nervous system and is characterized by major immune-mediated myelin and axonal damage or axonal loss explicable to the absence of myelin sheaths. Here we present the early findings of the gene expression study of myelinogenesis-related genes of MS rat models which were treated with a novel protocol of taper up-off of opium tincture.

The study included normal Lewis rats, MS rat models by induction of experimental autoimmune encephalomyelitis (EAE) without treatment, and MS rat models with a novel protocol of taper up-off treatment of opium tincture called Dezhakam-step-time (DST) in different dosages. RNA was extracted and cDNA was synthesized from the spinal cord tissue. Gene expression analysis was conducted for eight genes as markers of myelinogenesis (\textit{OLIG1, OLIG2, MBP, MYRF, PLP1, PMP22, EGF, and UGT8}) using the Real time PCR.

All eight genes were down-regulated in EAE models vs. healthy controls and all eight genes were up-regulated after the taper up-off treatment of opium tincture. The most over-expression of myelinogenesis-related genes was revealed at higher dosages of opium tincture.

These are the early results of a gene expression study in a multiple sclerosis model treated with opium tincture. It seems that the opium tincture method may induce the activation of myelinogenesis in EAE models which could lead to a potential treatment for improvement of neural dysfunctions in MS patients.

Introduction

Multiple sclerosis (MS) is an autoimmune and demyelinating disorder (1) with enigmatic and controversial pathogenesis. While the humoral immune system and B cells and their associated antibodies are playing an important role in the pathogenesis of MS, it is mainly a T-cell-mediated autoimmune disease (2). The main symptoms of MS are related to major demyelination and axonal loss along with short-term loss of nerve conduction and circulating factors (3).

Destruction of myelin sheaths and the myelin-producing oligodendrocytes (ODCs) along with the remyelination failure had been reported in MS (4). It has been reported that epidermal growth factor (EGF) and normal prions are significantly reduced in the central nervous system (CNS) in MS and EGF administration could prevent demyelination and inflammatory reactions. On the other hand, cobalamin levels decrease in the spinal cord of MS patients (5).

\textit{OLIG1} and \textit{OLIG2} genes are the main members of the gene family that encodes basic helix-loop-helix transcription factors that are expressed in the spinal cord and developing and mature central nervous system (CNS). \textit{OLIG} proteins regulate cellular specification and differentiation in CNS including the development of oligodendrocytes and neural cells, and astrocyte specification (6). \textit{OLIG1} is a central regulator of oligodendrocyte myelinogenesis and axonal recognition. \textit{OLIG1} is involved in the
transcription regulation of most important myelin-specific genes, including MBP, PLP1, and MAG, and down the regulation of the GFAP gene which is a major astrocyte-specific gene (7). Lack of the OLG1 gene could cause downregulation of myelin-specific genes, and OLG1-null mice models show general progressive axonal degeneration and gliosis (8). OLG2 is also a transcription factor that activates the expression of myelin-associated genes in the oligodendrocyte-lineage cells which in turn will lead to myelination (9).

Ciliary neurotrophic factor (CNTF) plays a major role in induction of promyelinating mechanism (10). CNTF is an important survival factor for oligodendrocytes which protects oligodendrocytes from several death signals (11), along with effects in the maturation of oligodendrogial progenitor cells to mature myelin-forming cells, and myelinogenesis (12).

Experimental autoimmune encephalomyelitis (EAE) is the most accepted animal model of MS that can be induced by inoculation of susceptible animals with a range of CNS antigens including myelin-oligodendrocyte glycoprotein (MOG). EAE would cause infiltration of the spinal cord and nerve roots with inflammatory cells that in turn would lead to demyelination of axons and axonal damages (13) along with glutamate toxicity and dysregulation of ion channels including calcium channels (14).

Gene expression profiling conducted on EAEs induced with myelin-oligodendrocyte glycoprotein (MOG) in the spinal cords in mice and rats showed suppression of mRNA level of cholesterol biosynthesis and upregulation of immune-related molecules, extracellular matrix and cell adhesion molecules (15) (16).

The present study was carried out to evaluate the gene expression pattern alterations of the multiple sclerosis model of Lewis rat (MOG-EAE), treated with a novel protocol of taper up-off of opium tincture, to understand the potential effects of opium on Multiple sclerosis. This paper is a short report of the early results of the study which focuses on the expression level of melanogenesis-related genes in experiment and control groups.

**Methods & materials**

**Animal Modeling of Multiple sclerosis by induction of Experimental autoimmune encephalomyelitis (EAE):**

Rodents were kept in an animal laboratory with regulated 12-hour light/12-hour dark cycles, with a climate-controlled environment and pathogen-free polystyrene cages designed for standard access to rodent chow and water. Based on previous studies and standard animal modeling of Multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) as a model of autoimmune central nervous system diseases has been used for modeling of demyelinating multiple sclerosis (MS) via injection of myelin oligodendrocyte glycoprotein (MOG) (MOG92-106) (17). EAE was induced in 11-week-old male and female Lewis rats by subcutaneous injection at the base of the tail of 50 µg recombinant MOG, using
standard methods based on previous studies. Healthy unimmunized rats with matched age weight and gender were used as controls. The list and description of groups are shown in Table 1.

<table>
<thead>
<tr>
<th>Group name</th>
<th>Description</th>
<th>Number of sacrificed rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>EAE model treated with dose 1 opium tincture</td>
<td>six female/six male</td>
</tr>
<tr>
<td>G2</td>
<td>EAE model treated with dose 2 opium tincture</td>
<td>six female/five male</td>
</tr>
<tr>
<td>G3</td>
<td>EAE model treated with dose 3 opium tincture</td>
<td>six female/six male</td>
</tr>
<tr>
<td>G4</td>
<td>EAE model treated with dose 4 opium tincture</td>
<td>six female/six male</td>
</tr>
<tr>
<td>Multiple sclerosis model (EAE)</td>
<td>EAE model with no treatment</td>
<td>five female/six male</td>
</tr>
<tr>
<td>Normal control</td>
<td>Healthy rats with no treatment</td>
<td>six female/six male</td>
</tr>
<tr>
<td>Sham</td>
<td>Normal rats with force-feeding of water</td>
<td>six female/five male</td>
</tr>
</tbody>
</table>

EAE: experimental autoimmune encephalomyelitis

Treatment with taper up-off treatment of opium tincture:

We developed a laboratory-designed method of taper up-off treatment called the Dezhakam-step-time (DST) method, which starts Opium tincture dosage intake from the lowest dose on the first day and the dose will increase with a 20% rate (or 0.8 coefficient) for each oral feeding (two times per day), until 18 oral feeding (9 days). Then the dosage of feeding is reduced by the same 20% rate (or 0.8 coefficient) for the next 18 oral feeding (9 days). The Rats will take the same dosage in first time and last time of oral feeding. A list of groups and descriptions of each group are presented in Table 1. Four sets of dosages were used in four experiment groups. In the present study dose one protocol started with 2.35 mg/kg Force-feeding and increased with 0.8 coefficient until 65.58 mg/kg in 18th oral feeding time. Doses two, three, and four started at 3.52 mg/kg, 4.7 mg/kg, and 5.87 mg/kg respectively.

Scarification and tissue collection

All rats were sacrificed on the same day at the standard animal laboratory. A sixty-milliliter syringe with a large bore blunt-ended needle is used to excise the spinal cord from the spinal column. All euthanasia processes were monitored by the researchers until confirmation of euthanasia accomplishment. Housing and euthanasia procedures are conducted based on the ARRIVE55 Guidelines checklist and protocols of previous studies (18) (19).

RNA Extraction
Total RNA was extracted from tissues using an RNA Purification kit (GeneJET™ RNA Purification Kit#K0732, Thermo scientific - Fermentas, Latvia) as per the manufacturer instructions and treated with DNase Treatment & Removal Reagents (DNase I, RNase-free (#EN0521) Fermentas, Latvia), according to the manufacture protocol to total removal of all traces of genomic DNAs. Quantity of extracted RNAs assessed by Nanodrop-1000 equipment and of RNAs quality check carried out using the BioRad Experion automated gel electrophoresis system (BioRad Laboratories Inc.) All extracted samples were restored in freezer storage at −80°C before further procedures.

**Synthesis of cDNA:**

Copied DNA synthesized using the transcription first strand cDNA Synthesis Kit (RevertAid Premium First Strand cDNA Synthesis Kit #K1652, Thermo Scientific -Fermentas, Latvia) based on previous studies and protocol of Thermo Scientific company.

**Gene expression analysis:**

The expression level of targeted genes (gene list presented in Table 2) was evaluated by Quantitative Real time PCR. In the beginning specific primers and probes had been designed using "GenScript Real-time PCR (TaqMan) Primer Design " software and checked by blasting on the NCBI website. The RNA samples of randomly chosen control rats used for serial dilutions (1: 4) of pooled cDNA aimed to draw the standard curves. The CFX96 Touch Real-Time PCR Detection System (BIO-RAD, California, United States) is used for Quantitative Real Time-PCR with the triplicate method. Having no signal in no-template control samples along with an $R^2$ value more than 0.99 in the standard curve were two quality-checking criteria of Real-Time PCR analysis. The PCR reaction efficacy was calculated using an online software called Lin-Reg PCR (Amsterdam, Netherlands). TaqMan® PCR Starter Kit, Thermo Scientific - Fermentas, Latvia) used for all samples. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a housekeeping gene for normalization. Ratio calculation carried out by Livak formula.
Table 2
list of studied genes

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Entrez gene name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olig1</td>
<td>oligodendrocyte transcription factor 1</td>
<td>It is involved in neuron differentiation and regulation of transcription by RNA polymerase II.</td>
</tr>
<tr>
<td>Olig2</td>
<td>oligodendrocyte transcription factor 2</td>
<td>It is a major regulator of ventral neuroectodermal progenitor cell fate.</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
<td>It is a major constituent of the myelin sheath of oligodendrocytes in the nervous system.</td>
</tr>
<tr>
<td>Myrf</td>
<td>myelin regulatory factor</td>
<td>It affects myelin production and regulation of myelin gene expression</td>
</tr>
<tr>
<td>Plp1</td>
<td>proteolipid protein 1</td>
<td>This gene encodes a transmembrane proteolipid protein which is the component of myelin.</td>
</tr>
<tr>
<td>Pmp22</td>
<td>peripheral myelin protein 22</td>
<td>This gene encodes an integral membrane protein which is a main component of myelin in the peripheral nervous system.</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
<td>This gene encodes a member of the epidermal growth factor superfamily and plays a major role in the growth, proliferation, and differentiation of numerous cells.</td>
</tr>
<tr>
<td>UGT8</td>
<td>UDP glycosyltransferase 8</td>
<td>The protein encoded by this gene belongs to the UDP-glycosyltransferase family that is involved in the biosynthesis of the myelin membrane of the central and peripheral nervous systems.</td>
</tr>
</tbody>
</table>

Statistical examinations:

Statistical assessments were performed with version 25 of SPSS. Normal distribution evaluation of all variables tested with Kolmogorov-Smirnov exam. One-way ANOVA analysis was used for statistical differences in multiple group comparisons. RNA integrity number, cDNA synthesis quality, plates/runs of qPCR, and primers and probe efficiency were defined as covariates, and the persistence of the significant difference between groups was evaluated by ANCOVA to control any potential confusion. Multiple comparisons corrections calculated Bonferroni correction statistical exam. The descriptive data are expressed as mean ± SD.

Results

During the treatment period one female rat from G2, one male rat from the multiple sclerosis model (EAE), and one female rat from the Sham group died before the scarification day. RNA quality analysis showed all samples had RNA quality indicator (RQI) values higher than 9.5. Results of gene expression assessments and comparisons between groups in differentially expressed genes are presented in Table 3. All genes were down-regulated in EAE models and up-regulated after the treatment in all four
experiment groups. The greatest increase in mRNA levels was seen in G3 and G4. No significant difference was detected in the expression level of genes between female and male rats in any group.

Table 3
mRNA level and statistical comparisons of gene expression evaluations

<table>
<thead>
<tr>
<th>Gene</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>MS model</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olig1</td>
<td>Ratio: 0.61</td>
<td>Ratio: 0.66</td>
<td>Ratio: 0.78</td>
<td>Ratio: 0.83</td>
<td>Ratio: 0.51</td>
<td>Ratio: 0.94</td>
</tr>
<tr>
<td></td>
<td>P value:0.008</td>
<td>P value:0.01</td>
<td>P value:0.08</td>
<td>P value:0.19</td>
<td>P value:0.006</td>
<td>P value:0.28</td>
</tr>
<tr>
<td>Olig2</td>
<td>Ratio: 0.69</td>
<td>Ratio: 0.73</td>
<td>Ratio: 0.82</td>
<td>Ratio: 0.86</td>
<td>Ratio: 0.42</td>
<td>Ratio: 0.91</td>
</tr>
<tr>
<td></td>
<td>P value:0.008</td>
<td>P value:0.01</td>
<td>P value:0.07</td>
<td>P value:0.12</td>
<td>P value:0.001</td>
<td>P value:0.77</td>
</tr>
<tr>
<td>MBP</td>
<td>Ratio: 0.73</td>
<td>Ratio: 0.78</td>
<td>Ratio: 0.88</td>
<td>Ratio: 0.85</td>
<td>Ratio: 0.67</td>
<td>Ratio: 0.96</td>
</tr>
<tr>
<td></td>
<td>P value:0.006</td>
<td>P value:0.01</td>
<td>P value:0.1</td>
<td>P value:0.13</td>
<td>P value:0.004</td>
<td>P value:0.35</td>
</tr>
<tr>
<td>Myrf</td>
<td>Ratio: 0.68</td>
<td>Ratio: 0.75</td>
<td>Ratio: 0.91</td>
<td>Ratio: 0.93</td>
<td>Ratio: 0.59</td>
<td>Ratio: 0.96</td>
</tr>
<tr>
<td></td>
<td>P value:0.006</td>
<td>P value:0.01</td>
<td>P value:0.15</td>
<td>P value:0.21</td>
<td>P value:0.004</td>
<td>P value:0.68</td>
</tr>
<tr>
<td>Plp1</td>
<td>Ratio:0.59</td>
<td>Ratio:0.64</td>
<td>Ratio:0.83</td>
<td>Ratio:0.94</td>
<td>Ratio:0.48</td>
<td>Ratio:1.06</td>
</tr>
<tr>
<td></td>
<td>P value:0.008</td>
<td>P value:0.009</td>
<td>P value:0.07</td>
<td>P value:0.1</td>
<td>P value:0.002</td>
<td>P value:0.29</td>
</tr>
<tr>
<td>Pmp22</td>
<td>Ratio:0.7</td>
<td>Ratio:0.77</td>
<td>Ratio:0.84</td>
<td>Ratio:0.9</td>
<td>Ratio:0.53</td>
<td>Ratio:0.9</td>
</tr>
<tr>
<td></td>
<td>P value:0.01</td>
<td>P value:0.03</td>
<td>P value:0.12</td>
<td>P value:0.17</td>
<td>P value:0.004</td>
<td>P value:0.47</td>
</tr>
<tr>
<td>EGF</td>
<td>Ratio: 0.77</td>
<td>Ratio: 0.83</td>
<td>Ratio: 0.82</td>
<td>Ratio:88</td>
<td>Ratio: 0.71</td>
<td>Ratio: 0.92</td>
</tr>
<tr>
<td></td>
<td>P value:0.06</td>
<td>P value:0.09</td>
<td>P value:0.11</td>
<td>P value:0.15</td>
<td>P value:0.006</td>
<td>P value:0.3</td>
</tr>
<tr>
<td>UGT8</td>
<td>Ratio: 0.73</td>
<td>Ratio: 0.78</td>
<td>Ratio: 0.84</td>
<td>Ratio:92</td>
<td>Ratio: 0.62</td>
<td>Ratio: 1.1</td>
</tr>
<tr>
<td></td>
<td>P value:0.009</td>
<td>P value:0.01</td>
<td>P value:0.04</td>
<td>P value:0.08</td>
<td>P value:0.005</td>
<td>P value:0.2</td>
</tr>
</tbody>
</table>

MS: multiple sclerosis, OLIG1: oligodendrocyte transcription factor 1, OLIG2: oligodendrocyte transcription factor 2, MBP: myelin basic protein, MYRF: myelin regulatory factor, PLP1: proteolipid protein 1, PMP22: peripheral myelin protein 22, EGF: epidermal growth factor, UGT8: UDP glycosyltransferase 8

Discussion
The molecular pathways leading to disease severity progression and remyelination failure or success in MS remain largely unknown (20). While the molecular mechanisms of melanogenesis and myelin gene activation and inactivation are not completely clarified, finding a potential way to activating of melanogenesis in MS patients, could lead to a potential treatment for neural loss of functions in CNS.

Our early results showed, that several markers of myelination (21) are down-regulated in the spinal cord during EAE and significantly raised after the force-feeding of opium tincture. Some of these genes are related to each other in several signaling pathways and contribute to neuronal plasticity. Interestingly, our results on the up-regulation of myelogenesis markers weren't gender dependent which may lead to a potentially applicable treatment for both genders in MS.

Opium usage could enhance the antioxidant capacity and reduce the inflammation in the CNS which can reduce the extent of damage in cerebral ischemia (22). Opium contains about frothy alkaloids and more than seventy components including several sugars and organic acids (23). Some opioid-based analgesics such as morphine are associated with immunosuppressive effects on innate immunity, whilst having diverse effects on adaptive immunity (24). It seems that using the whole alkaloid content of opium in a special taper-up-off protocol may induce anti-inflammation effects which in turn could lead to the gradual reactivation of myelogenesis that has been reduced dramatically during the MS symptoms advancement. The mechanism and mode of action of the DST method at the molecular level are not completely clarified, but it seems that opium, with several alkaloids, can help regulate upstream mechanisms of gene expression and epigenetics, especially transcription factors.

Previous studies showed the myelination expression patterns alterations in the spinal cords of EAE mice models induced by estrogen (25). It has been suggested that signaling pathways that promote myelin formation and repair could enhance remyelination (20). To the best of our knowledge, the main reasons for remyelination failure in EAE models as well as human MS patients include calpain genes overexpression and activation of glycolipid-reactive iNKT cells and sphingoid-mediated inflammation. In addition, the treatments for MS patients are based on Cytokine-based immune intervention, antigen-based immunomodulation, and also recombinant monoclonal antibodies which induce remyelination.

Major attribution of EGF in myelin gene expression, myelin sheath compaction, and myelinogenesis had been reported in both peripheral and central nervous systems (26). Although the processes of molecular mechanisms underlying oligodendrocyte myelinogenesis are poorly defined the significant over-expression of OLIG genes is a reliable marker for myelinogenesis activation (27). Moreover, MYRF is required to initiate and maintain myelination, and upregulation of MYRF along with MBP, UGT8, PLP1, and PMP22 could orchestrate the remyelination in neurodegenerative disease which in turn may lead to the improvement of the circuit's functions in CNS (28). The neural circuit's normal functions need several types of neurons astrocytes and oligodendrocytes, at the appropriate periods and regions (29).

Our early results may lead to the development of a treatment strategy that induces myelination by altering the epigenetic pattern of related genes; which could apply to other severe neurodegenerative diseases with loss of the neuronal circuits such as Huntington's disease (HD), Parkinson's disease (PD),
and Amyotrophic Lateral Sclerosis (ALS) as well as MS. Therefore, the DST method of taper up-off of opium could improve the speed and efficiency of signal propagation.

Limitations:

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the central nervous system (CNS) that results in variable severities of neurodegeneration. While the early results of the present study were interesting, the myelination is not universally applied in the central nervous system and may vary in the spinal cord versus the brain in EAE models. Further results especially the whole genome expression profiling of samples may clarify our knowledge about the effects of opium usage with the DST method on the EAE model.

Declarations

Ethics Statement

This research was approved by the Central Ethical Committee of the Islamic Azad University with the approval number 38966.

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23. Shetge SA, Dzakovich MP, Cooperstone JL, Kleinmeier D, Redan BW. Concentrations of the opium alkaloids morphine, codeine, and thebaine in poppy seeds are reduced after thermal and washing treatments but are not affected when incorporated in a model baked product. Journal of agricultural and food chemistry. 2020;68(18):5241–8.


