NRC Research Press

ARTICLE

Effects of essential oil from *Chamaecyparis obtusa* on cytokine genes in the hippocampus of maternal separation rats

Hae Jeong Park, Su Kang Kim, Won Sub Kang, Jong-Min Woo, and Jong Woo Kim

Abstract: We investigated the effects of an essential oil from *Chamaecyparis obtusa* (EOCO) on early life stress, using maternal separation (MS) rats and a microarray method to analyze the changes in gene expressions caused by EOCO in the hippocampus of MS rats. Rats in the MS groups were separated from their respective mothers from postnatal day (pnd) 14 to 28. Rats in the EOCO-treated groups were exposed to EOCO for 1 or 2 h by inhalation from pnd 21 to 28. The EOCO-treated MS rats showed decreased anxiety-related behaviors compared with the untreated MS rats in the elevated plus-maze (EPM) test. In the microarray analysis, we found that EOCO downregulated the expressions of cytokine genes such as *Ccl2*, *Il6*, *Cxcl10*, *Ccl19*, and *Il1rl* in the hippocampus of MS rats, and also confirmed that using reverse transcriptase – PCR. In particular, the expressions of *Ccl2* and *Il6* were predominantly decreased by EOCO in the hippocampus of MS rats. Interestingly, protein expression was also reduced by EOCO in MS rats. These results indicate that EOCO decreases MS-induced anxiety-related behaviors, and modulates cytokines, particularly Ccl2 and Il6, in the hippocampus of MS rats.

Key words: Chamaecyparis obtusa, early-life stress, maternal separation, cytokine gene, anxiety-related behavior.

Résumé : Nous avons examiné l'effet de l'huile essentielle de *Chamaecyparis obtusa* (HECO) sur un stress de début de vie en utilisant la séparation maternelle (SM) chez le rat par l'analyse des changements d'expression génique induits par l'HECO dans l'hippocampe des rats SM sur des puces à ADN. Les rats des groupes SM ont été séparés de leur mère des jours postnataux (jpn) 14 à 28. Les rats des groupes traités à l'HECO ont été exposés pendant une ou deux heures par inhalation entre les jpn 21 et 28. Lors du test en labyrinthe en croix surélevée, les comportements anxieux étaient diminués chez les rats traités à l'HECO comparativement aux rats SM. L'analyse des puces à ADN a révélé que l'HECO régulait à la baisse l'expression de gènes de cytokines comme *Ccl2, Il6, Cxcl10, Ccl19* et *Il1rl* dans l'hippocampe des rats SM, régulation confirmé par RT–PCR. Notamment, l'expression de *Ccl2* et de *Il6* était diminuée de manière prédominante par l'HECO dans l'hippocampe des rats SM. Fait intéressant, l'expression des protéines correspondantes était aussi diminuée par l'HECO chez les rats SM. Ces résultats indiquent que l'HECO diminue les comportements anxieux induits par la SM et module l'expression des cytokines, notamment Ccl2 et IL-6, dans l'hippocampe des rats SM. [Traduit par la Rédaction]

Mots-clés : Chamaecyparis obtusa, stress de début de vie, séparation maternelle, gène de cytokine, comportement anxieux.

Introduction

Adverse early life experiences such as social isolation and parental loss often produce impulsive aggression and antisocial personality symptoms (Agid et al. 1999; Barnow et al. 2001). Such experiences have been associated with serious psychiatric problems, including depression and anxiety disorders (Agid et al. 1999; Barnow et al. 2001). Maternal separation (MS) in rats during the early postnatal period is a well-established animal model of early life stress (Veenema et al. 2006). It has also served as a model of psychopathology for anxiety disorders and depression (Kalinichev et al. 2002; Pryce et al. 2005). Indeed, MS rats have been characterized by increased anxiety-related behaviors (Huot et al. 2001; Kalinichev et al. 2002), and alterations of various neurobiological parameters have been shown during brain development of MS rats (Plotsky and Meaney 1993; Wilber et al. 2009).

Previous studies have shown stress relaxing effects from forest bathing, or phytoncides, which are aromatic essential oils emitted mainly by trees, of which the major ingredients are organic compounds called terpenoids (Hong et al. 2004; Li et al. 2009, 2010). Indeed, forest bathing and exposure to phytoncides decreased the stress hormone levels in human urine (Li et al. 2009; Li and Kawada 2011). In addition, phytoncides reduced stress-induced elevation of blood pressure in stroke-prone spontaneously hypertensive rats (Kawakami et al. 2004). Moreover, phytoncides modulated neural transmission in the brain by increasing the potentiation of $GABA_A$ receptors, which were involved in anxiolytic, anticonvulsant, anesthetic, and sedative activities (Aoshima and Hamamoto 1999).

Chamaecyparis obtusa (commonly known as Hinoki), which belongs to the Cupressaceae family, is a tropical tree species found in Japan and the southern region of South Korea. Essential oils extracted from *C. obtusa* and other Cupressaceae family plants contain several types of terpenes including limonene, bornyl acetate, α-terpineol, and elemol (Sullivan et al. 1994), which are the active components that exert antigastropathic, anti-inflammatory, and antioxidant activity. Indeed, it has been shown that essential oils from Cupressaceae, including *C. obtusa*, have antibacterial, antifungal, and anti-cancer effects (Hong et al. 2004; Li et al. 2009;

Received 24 June 2013. Accepted 10 October 2013.

H.J. Park and S.K. Kim. Kohwang Medical Research Institute, School of Medicine, Kyung Hee University, Seoul, Republic of Korea.
W.S. Kang and J.W. Kim. Department of Neuropsychiatry, School of Medicine, Kyung Hee University, 26 Kyunghee-daero, Dongdaemun-gu, Seoul 130-701, Republic of Korea.
J.M. Woo. Department of Psychiatry, Seoul Paik Hospital, Inje University, School of Medicine, Seoul, Republic of Korea; Stress Research Institute, Inje University, Seoul, Republic of Korea.
Corresponding author: Jong Woo Kim (e-mail: hjpark17@gmail.com).

Published at www.nrcresearchpress.com/cjpp on 24 October 2013.

Ho et al. 2012). In addition, a clinical study has reported that inhalation of essential oil from *C. obtusa* (EOCO) decreased the concentrations of stress hormones such as adrenaline and noradrenaline in humans (Li et al. 2009). A recent study has also reported on the action of EOCO on the central nervous system (CNS), showing that inhalation of EOCO ameliorated the impairment of cognitive function and neuronal cell death induced by injection of β-amyloid in rats (Bae et al. 2012).

Considering the effects of forest bathing, phytoncides, and EOCO on stress relaxation, and the activity of EOCO on CNS, we speculated that EOCO may have therapeutic effects against psychiatric problems caused by stress. In this study, we investigated the effect of EOCO on early life stress using the MS animal model. To detect the activity of EOCO, anxiety-related behaviors were analyzed, and changes in gene expressions caused by EOCO were studied in the hippocampus of MS rats using a microarray method.

Materials and methods

Animals and maternal separation

Timed-pregnant Sprague–Dawley rats were provided on gestation day 17 from Daehan BioLink (DBL; Seoul, South Korea). The rats were individually housed in standard rat cages, and maintained under a 12 h (light) – 12 h (dark) cycle at a standard temperature (22 ± 3 °C) with food and water freely available.

The day of delivery was designated as postnatal day (pnd) 0. On pnd 14, pups were randomly assigned to one of 5 groups ($n = 10$ per group): (*i*) maternal care (MC), (*ii*) 2 h EOCO-treated MC, (*iii*) MS, (*iv*) 1 h EOCO-treated MS, or (*v*) 2 h EOCO-treated MS. For validating the effect of EOCO on anxiety-related behaviors in MS rats, a different set of pups were also assigned to one of 4 groups ($n = 7$ per group) on pnd 14: (*i*) saline-treated MC, (*ii*) fluoxetine-treated MC, (*iii*) saline-treated MS, or (*iv*) fluoxetine-treated MS (Baek et al. 2012). Pups in the MC groups were housed with their mothers under standard conditions. MS was conducted using the method described by Lee et al. (2001), totally separating from pups' respective mothers from pnd 14 (Park et al. 2011; Baek et al. 2012). Those in the MS groups were maintained individually for 14 days (pnd 14–28) in a new cage with free access to food and water. All animal experiments were conducted in accordance with the animal care guidelines of the National Institute for Health (NIH) and the Korean Academy of Medical Sciences.

Drugs and treatments

EOCO produced from the stem and leaf of *C. obtusa* was purchased from a commercial source (Yesco Service Co., Ltd., Seoul, South Korea). EOCO was analyzed using gas chromatography – mass spectrometry at the Korea Testing & Research Institute (KTRI; Gunpo, South Korea). EOCO comprised α-pinene, β-pinene, β-phellandrene, terpinyl acetate, δ-3-carene, limonene, p-cimene, camphene, α-terpinolene, and others.

EOCO treatment was administered via inhalation according to the method from Seol et al. (2010), with modifictions. The inhalation apparatus consisted of a square transparent Plexiglas cage (50 cm × 60 cm × 50 cm) with a hole in the bottom. For efficient vaporization, 20 mL of warm water was poured into a Petri dish placed in the bottom of the cage. In the EOCO-treated groups, 1 mL of EOCO was added to the water. Rats of the EOCO-treated groups were exposed to EOCO once a day for 1 h (1 h EOCO-treated MS group) or 2 h (2 h EOCO-treated MC, and 2 h EOCO-treated MS groups) for 7 days (pnd 21–28). For 7 days, the mean volume of EOCO vaporization per day in each cage was 16 μL/cage in 1 h-EOCO exposure, and 27 μL/cage in 2 h-EOCO exposure. Rats in the EOCO-nontreated groups (MC and MS groups) were exposed to warm water only, as an odorless condition using the same schedule and method.

For validating the effects of EOCO on anxiety-related behavior in MS rats, the anti-depressant fluoxetine (5 mg/kg; Sigma–Aldrich, St. Louis, Missouri, USA) was administered via subcutaneous injection once a day to the fluoxetine-treated MC and fluoxetine-treated MS groups, for 7 days (pnd 21–28) (Baek et al. 2012). Saline was also subcutaneously injected in the saline-treated MC and saline-treated MS group rats using the same schedule.

Elevated plus-maze (EPM) test

On pnd 27, the EPM test was performed to determine anxiety-related behavior. The plus-maze was constructed from black wood and elevated to a height of 50 cm. It consisted of 2 open arms (30 cm × 10 cm) and 2 enclosed arms (30 cm × 10 cm × 30 cm, with an open roof). After being habituated to the testing room for at least 20 min, each rat was placed in the center of a cross maze facing an open arm. Time spent and the number of entries into the open or closed arms were recorded during a 5 min test session. An entry was defined as 2 forepaws placed on the arm. The percentage of time spent in the open arms and the percentage of open arm entries were measured as parameters of anxiety-related behavior.

Tissue collection

Rats from all of the groups were sacrificed on pnd 28. We dissected out the hippocampus of each rat, weighed them and kept them frozen for microarray analysis and enzyme-linked immunosorbent assay (ELISA).

Gene expression microarray analysis

Gene expression was analyzed using the Agilent Rat Gene Expression 4× 44K (V3) Microarray (Agilent Technology, Palo Alto, California, USA). Total RNA from isolated hippocampus was extracted using TRIzol reagent (Invitrogen, Carlsbad, Calif.). For extracted RNA, target cRNA probes were synthesized, and hybridized to microarray using the Low Input QuickAmp Labeling Kit (Agilent Technology) according to the manufacturer's instructions. Hybridized microarrays were then scanned with the Agilent DNA microarray Scanner (Agilent Technology).

Data quantifications were performed using Agilent Feature Extraction software 9.3.2.1 (Agilent Technology). The average fluorescence intensity for each spot was calculated and the local background was subtracted. All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3.1 (Agilent Technology). Genes were filtered and flag-out genes were removed in each experiment. Intensity-dependent normalization (Lowess) was performed, where the ratio was reduced to the residual of the Lowess fit of the intensity versus ratio curve. Averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity.

Functional annotation of genes was performed according to the Gene Ontology™ Consortium (http://www.geneontology.org/index.shtml) by GeneSpringGX 7.3.1.

RT–PCR analysis

Reverse transcriptase – polymerase chain reaction (RT–PCR) was performed to confirm the results obtained in the microarray analysis. We selected 5 cytokine genes [chemokine (C-C motif) ligand 2 (*Ccl2*), interleukin 6 (*Il6*), chemokine (C-X-C motif) ligand 10 (*Cxcl10*), *Ccl19*, interleukin 1 receptor-like 1 (*Il1rl1*)] that were significantly downregulated in the EOCO-treated MS group compared with the MS group in microarray analysis. The PCR reaction was conducted using first-strand cDNA, and each gene specific primer at each annealing temperature (Table 1). RT–PCR products were electrophoresed in a 1.5% agarose gel and visualized using ethidium bromide staining.

Enzyme-linked immunosorbent assay

The hippocampus isolated from each group was homogenized in RIPA buffer. The levels of Ccl2 and Il6 were detected in homogenized tissues using ELISA according to the manufacturer's protocol (R & D Systems, Minneapolis, Minnesota, USA). Capture

Table 1. Sequences of the primers used in RT–PCR analysis.

Gene	Primer sequence (sense/antisense)	Size (bp)	Temp. (°C)
Ccl2	5′-AGGTGTCCCAAAGAAGCTGT-3′ 5′-TGCTTGAGGTGGTTGTGGAA-3′	241	61
Il6	5′-TGTGCAATGGCAATTCTGAT-3′ 5′-TGGTCTTGGTCCTTAGCCAC-3′	363	58
Cxcl10	5′-GCTTATTGAAAGCGGTGAGC-3′ 5′-ATTTGCCATCTCACCTGGAC-3′	462	62
Ccl19	5′-CTTCAGCCTGCTGGTTCTCT-3′ 5′-TGCTCACACTCACGTTCACA-3′	429	58
Il1rl1	5′-ACATCAACCGCCTAGTGGAC-3′ 5′-AGGGATTTTGCAGTTTGGTG-3′	440	61
Actb	5′-TGTCACCAACTGGGACGATA-3′ 5′-TCTCAGCTGTGGTGGTGAAG-3′	392	58

antibodies for Ccl2 and Il6 (R & D Systems) were added to each well of 96-well plates (Nunc, Roskilde, Denmark), and incubated overnight at 4 °C. The plates were washed in PBS with 0.05% Tween 20 (Sigma–Aldrich). The plates were blocked with PBS containing 1% BSA and 0.05% NaN_3. Samples and Ccl2/Il6 standards (R & D Systems) were added to each well, and the plates were incubated for 2 h at room temperature. After washing the plates, detection antibodies for Ccl2 and Il6 (R & D Systems) were added. After washing the plates, avidin HRP (R & D Systems) was added, and the plates were incubated for 20 min. Tetramethylbenzidine (TMB)/peroxidase substrate (R & D Systems) was added to each well, and the reaction was allowed to develop in the dark for 15 min. The reaction was stopped by adding 1 mol/L H_2SO_4 to each well, and the absorbance was read on a plate reader at 450 nm (Molecular Devices, Toronto, Ontario, Canada).

Statistical analyses

All values are the mean ± SEM. The differences among the groups were tested by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, using SPSS software (release 18.0; SPSS Inc., Chicago, Illinois, USA). In addition, the interaction between MS and EOCO or fluoxetine treatment was analyzed by 2-way ANOVA. A *p*-value of 0.05 was considered statistically significant.

Results

Effect of EOCO on anxiety-related behavior

An EPM test was performed to assess anxiety-related behavior. One-way ANOVA showed significant differences in the percentage of open arm entries ($F_{[4,45]} = 5.090$, $p = 0.002$) and the percentage of time spent in the open arms ($F_{[4,45]} = 8.023$, $p < 0.001$) among the 5 groups (MC, 2 h EOCO-treated MC, MS, 1 h EOCO-treated MS, and 2 h EOCO-treated MS). As shown in Fig. 1A, the MS rats had a significantly lower percentage of open arm entries than the MC rats ($p = 0.002$, Tukey's test). In comparison, the percentage of open arm entries in the 1 h ($p = 0.015$, Tukey's test) and 2 h EOCO-treated MS rats ($p = 0.009$, Tukey's test) increased compared with that in the MS rats. Additionally, the MS rats spent less time in the open arms compared with the MC rats ($p < 0.001$, Tukey's test), whereas the EOCO-treated MS rats spent more time in the open arms than the MS rats ($p = 0.007$ in 1 h EOCO-treated MS group vs. MS group and $p = 0.001$ in 2 h EOCO-treated MS group vs. MS group, Tukey's test; Fig. 1B). Moreover, 2-way ANOVA showed a significant effect of maternal care and EOCO treatment interaction ($F_{[4,45]} = 13.004$, $p = 0.001$ for open arm entries; $F_{[4,45]} = 9.711$, $p = 0.003$ for open arm time), indicating that MS decreased time spent and number of entries into the open arm, and that this effect was alleviated by EOCO.

These results were similar for behaviors shown in rats in the saline-treated MC, fluoxetine-treated MC, saline-treated MS, and fluoxetin-treated MS groups. Significant differences among these 4 groups were shown on the percentage of open arm entries ($F_{[3,24]} = 15.515$, $p < 0.001$) and the percentage of time spent in the open arms ($F_{[3,24]} = 6.096$, $p = 0.003$) (one-way ANOVA). The percentages of open arm entries ($p < 0.001$, Tukey's test), and time spent in the open arms ($p = 0.015$, Tukey's test) were decreased in the saline-treated MS rats compared with the saline-treated MC rats, whereas the percentages were significantly increased in the fluoxetin-treated MS rats compared with the saline-treated MS rats ($p = 0.004$ for open arm entries and $p = 0.006$ for time spent in the open arms, Tukey's test). The significant effect of maternal care and fluoxetine treatment interaction was also revealed (2-way ANOVA: $F_{[3,24]} = 9.269$, $p = 0.006$ for open arm entries; $F_{[3,24]} = 7.249$, $p = 0.013$ for time spent in the open arm). These results indicated that EOCO reduced anxiety-related behavior shown in the MS rats.

Altered gene expressions by EOCO in the hippocampus of MS rats

A microarray was performed to assess the gene expression altered by EOCO in the hippocampus of MS rats. First, we investigated the alteration in gene expression caused by MS. We selected genes for which expressions were upregulated or downregulated more than 1.5-fold by MS (1.5 ≤ fold change or 0.67 > fold change). MS increased the expressions of 161 genes, and decreased the expressions of 32 genes in the rat hippocampus (data not shown).

We also investigated the changes in gene expressions by EOCO in the MS rats. The effect of EOCO was examined in the hippocampus of MS rats inhaling EOCO for 2 h. The expressions of 58 genes were upregulated more than 1.5-fold in the EOCO-treated MS group compared with the MS group, whereas the expressions of 53 genes were downregulated more than 1.5-fold (data not shown). Among these 111 genes, we found that EOCO particularly altered the expressions of cytokine genes in the hippocampus of MS rats. As shown in Table 2, EOCO reduced the expressions of 10 genes including *Ccl26*, *Ccl2*, and *Ccl20* in the hippocampus of MS rats, and elevated the expressions of 4 genes including interferon-induced protein 44-like (*Ifi44l*), and chemokine (C-C motif) receptor 8 (*Ccr8*). Of these 14 genes, we focused on the expression changes in the *Ccl2*, *Il6*, *Cxcl10*, *Ccl19*, and *Il1rl1* genes. The expressions of these genes were upregulated more than 1.5-fold by MS, whereas they were downregulated by EOCO. The mRNA levels of *Ccl2*, *Il6*, *Cxcl10*, *Ccl19*, and *Il1rl1* were elevated 2.084-, 1.535-, 2.598-, 1.532-, and 1.508-fold, respectively, in the hippocampus of MS rats compared with MC rats. Conversely, the mRNA levels of these genes were decreased 0.383-, 0.408-, 0.485-, 0.487-, and 0.607-fold, respectively, in the hippocampus of EOCO-treated MS rats compared with the MS rats.

mRNA expressions of cytokine genes in the hippocampus of EOCO-treated MS rats

To confirm the microarray findings, cytokine genes regulated by EOCO in the hippocampus of MS rats were selected. The mRNA expressions of *Ccl2*, *Il6*, *Cxcl10*, *Ccl19*, and *Il1rl1* genes were assessed by RT–PCR, reproducing the microarray findings. The efficiency of the reaction was adjusted by *Actb* (also known as β-actin) amplification. Significant differences among 4 groups (MC, 2 h EOCO-treated MC, MS, and 2 h EOCO-treated MS) were observed in the expressions of *Ccl2* ($F_{[3,8]} = 16.825$, $p = 0.001$), *Il6* ($F_{[3,8]} = 19.914$, $p < 0.001$), *Cxcl10* ($F_{[3,8]} = 6.850$, $p = 0.013$), *Ccl19* ($F_{[3,8]} = 18.294$, $p = 0.001$), and *Il1rl1* genes ($F_{[3,8]} = 170.236$, $p < 0.001$) (one-way ANOVA). As shown in Fig. 2, the mRNA expressions of *Ccl2* (2.55-fold), *Il6* (1.86-fold), *Cxcl10* (1.66-fold), *Ccl19* (1.55-fold), and *Il1rl1* genes (1.89-fold) were increased in the hippocampus of MS rats compared with the MC rats ($p = 0.001$ for *Ccl2*, $p = 0.001$ for *Il6*, $p = 0.021$ for *Cxcl10*, $p = 0.002$ for *Ccl19*, and $p < 0.001$ for *Il1rl1*, Tukey's test), whereas their expressions were attenuated in the hippocampus of EOCO-treated MS rats compared with the MS rats [*Ccl2*, 0.50-fold ($p = 0.004$); *Il6*, 0.54-fold ($p = 0.001$); *Cxcl10*, 0.65-fold ($p = 0.038$); *Ccl19*, 0.65-fold ($p = 0.002$); *Il1rl1*, 0.60-fold ($p < 0.001$)]. The 2-factor

Fig. 1. Effect of essential oil from *Chamaecyparis obtusa* (EOCO) on the anxiety-related behavior of maternal separation (MS) rats using the elevated plus-maze (EPM) test. Total open and closed arm entries in the EPM during a 5 min test were measured. Anxiety-related behavior is indicated by the percentage of time spent in the open arms (A) and the percentage of open arm entries (B). The EPM test was performed in maternal care (MC) and MS rats treated with or without EOCO for 1 h or 2 h (■), and was also used to validate the effect of EOCO in MC and MS rats treated with saline or fluoxetine (□). Data are the mean ± SEM. EOCO treated rats: *, $p < 0.05$ for the MS group compared with the the MC group; †, $p < 0.05$ for the EOCO-treated MS group compared with the MS group. Fluoxetine-treated rats: ‡, $p < 0.05$ for the saline-treated MS group compared with the saline-treated MC group; §, $p < 0.05$ for the fluoxetine-treated MS group compared with the saline-treated MS group; one-way ANOVA followed by Tukey's post-hoc test.

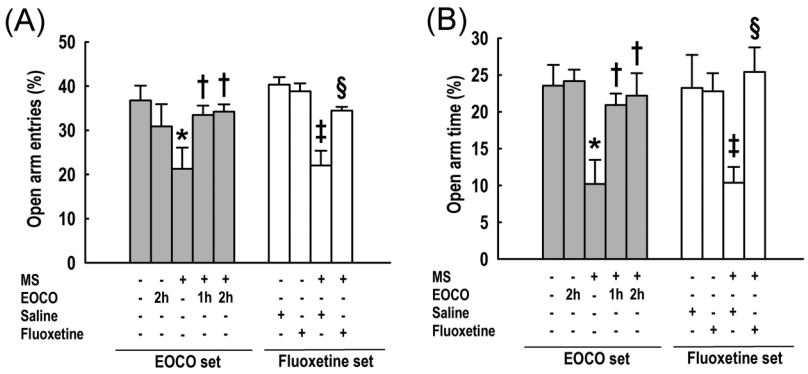


Table 2. Cytokine genes changed by essential oil of *Chamaecyparis obtusa* in the hippocampus of maternal separation rats.

Gene	Location	Accession	Description	Fold change	
				MS/MC	MS+EOCO/MS
Ccl26	12q12	NM_001109488	Chemokine (C-C motif) ligand 26	1.089	0.287
Ccl2	10q26	NM_031530	Chemokine (C-C motif) ligand 2	**2.084**	**0.383**
Ccl20	9q34	NM_019233	Chemokine (C-C motif) ligand 20	0.564	0.396
Il6	4q11	NM_012589	Interleukin 6	**1.535**	**0.408**
Cxcl10	14p22	NM_139089	Chemokine (C-X-C motif) ligand 10	**2.598**	**0.485**
Ccl19	5q22	NM_001108661	Chemokine (C-C motif) ligand 19	**1.532**	**0.487**
Ccr1	8q32	NM_020542	Chemokine (C-C motif) receptor 1	0.460	0.569
Cxcl3	14p22	NM_138522	Chemokine (C-X-C motif) ligand 3	0.410	0.594
Il1rl1	9q21	NM_001127689	Interleukin 1 receptor-like 1	**1.508**	**0.607**
Cxcr6	8q32	NM_001102587	Chemokine (C-X-C motif) receptor 6	0.705	0.633
Csf3	10q31	NM_017104	Colony stimulating factor 3	1.206	1.582
Tgfbrap1	9q22	NM_001106907	Transforming growth factor, beta receptor associated protein 1	1.729	1.984
Ccr8	8q32	ENSRNOT00000029766	Chemokine (C-C motif) receptor 8	1.239	2.858
Ifi44l	2q45	XM_227820	Interferon-induced protein 44-like	2.780	4.115

Note: Bold characters indicate the fold-change of cytokine genes upregulated (with decreasing in the MS rats compared to the MC rats) or downregulated (with increasing in the MS rats compared to the MC rats) in the 2 h EOCO-treated MS rats compared to the MS rats. MC, maternal care; MS, maternal separation; EOCO, essential oil of *Chamaecyparis obtuse.*

interaction effect of maternal care and EOCO treatment was also significant on the expression levels of these genes (2-way ANOVA: $F_{[3,8]} = 14.800$, $p = 0.005$ for *Ccl2*; $F_{[3,8]} = 20.441$, $p = 0.002$ for *Il6*; $F_{[3,8]} = 5.929$, $p = 0.041$ for *Cxcl10*; $F_{[3,8]} = 13.541$, $p = 0.006$ for *Ccl19*; $F_{[3,8]} = 184.092$, $p < 0.001$ for *Il1rl1*).

Protein levels of Ccl2 and Il6 in the hippocampus of EOCO-treated MS rats

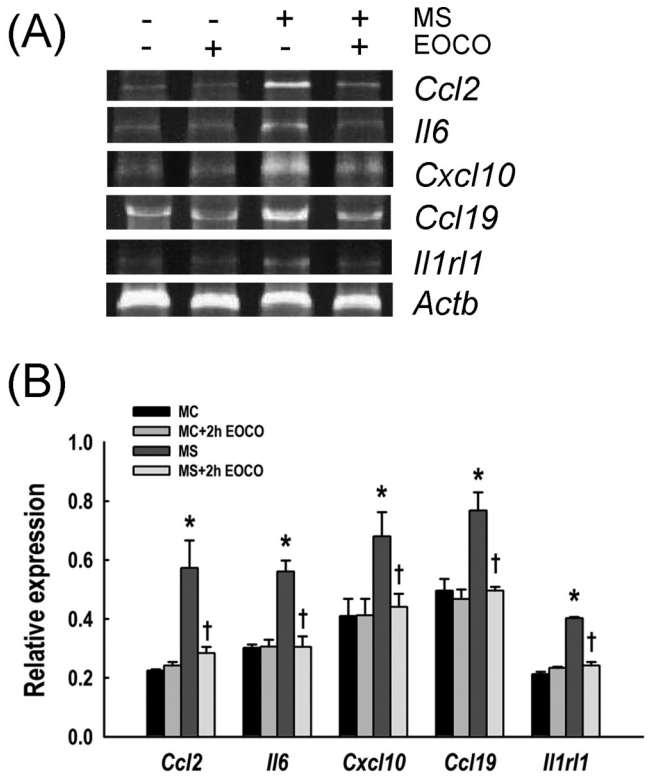
We measured the protein expressions of Ccl2 and Il6 using ELISA in the rat hippocampus. One-way ANOVA revealed significant differences in the levels of Ccl2 ($F_{[4,13]} = 14.402$, $p < 0.001$) and Il6 ($F_{[4,13]} = 17.859$, $p < 0.001$) among 5 groups (MC, 2-h EOCO-treated MC, MS, 1 h EOCO-treated MS, and 2 h EOCO-treated MS). The level of Ccl2 was 94.6 ± 2.7 pg/mL in the hippocampus of MC rats, and 106.3 ± 12.3 pg/mL in the hippocampus of 2 h EOCO-treated MC rats (Fig. 3A). The level of Ccl2 was increased in the MS rats (200.0 ± 9.4 pg/mL) compared with the MC rats ($p < 0.001$, Tukey's test), whereas it was time-dependently decreased in the hippocampus of EOCO-treated MS rats compared with the MS rats. The level of Ccl2 was 139.9 ± 9.9 in the 1 h EOCO-treated MS rats, and 118.7 ± 14.6 pg/mL in the 2 h EOCO-treated MS rats ($p = 0.010$ and $p = 0.001$, respectively, by comparison with the MS group, Tukey's test). As shown in Fig. 3B, the level of Il6 was also increased in the hippocampus of MS rats (471.0 ± 39.8 pg/mL) compared with the MC rats (168.2 ± 1.9 pg/mL; $p < 0.001$, Tukey's test). In comparison, it was attenuated in the hippocampus of rats in both the 1 h (311.8 ± 36.5 pg/mL) and 2 h EOCO-treated MS groups (304.3 ± 18.8 pg/mL) compared with the MS rats ($p = 0.009$ and $p = 0.007$, respectively, Tukey's test). Two-way ANOVA also showed a significant effect of maternal care and EOCO treatment interaction ($F_{[4,13]} = 16.771$, $p = 0.001$ for Ccl2; $F_{[4,13]} = 8.525$, $p = 0.012$ for Il6), indicating that MS increased levels of Ccl2 and Il6, and that these effects were alleviated by EOCO.

Discussion

In this study, EOCO reduced anxiety-related behaviors shown in MS rats, and decreased the mRNA expressions of cytokine genes such as *Ccl2*, *Il6*, *Cxcl10*, *Ccl19*, and *Il1rl1*, and the protein levels of Ccl2 and Il6 in the hippocampus of MS rats.

Previous studies have shown that MS increases anxiety-related behaviors, reducing the frequency of open arm entries and the

Fig. 2. Expression changes in cytokine genes caused by essential oil from *Chamaecyparis obtusa* (EOCO) in the hippocampus of maternal separation (MS) rats. (A) To confirm microarray results, the mRNA expressions of 5 cytokine genes, *Ccl2*, *Il6*, *Cxcl10*, *Ccl19*, and *Il1rl1* were analyzed using RT–PCR with total RNA from the hippocampus of the maternal care (MC), EOCO-treated MC, MS, and EOCO-treated MS rats. (B) For quantification of the RT–PCR results, the expression level of each gene was measured as optical density. *Actb* was amplified as an internal control. Results are the mean ± SEM. Experiments were repeated 3 times using the same samples; *, $p < 0.05$ for the MS group compared with the MC group; †, $p < 0.05$ for the EOCO-treated MS group compared with the MS group; one-way ANOVA followed by Tukey's post-hoc test.



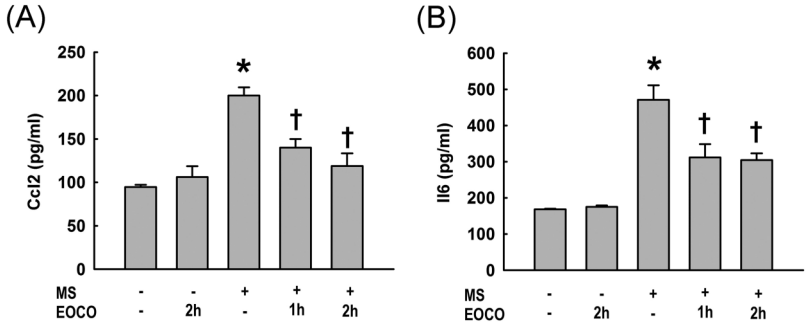
amount of time spent in the open arms in the EPM test by various MS protocols including the MS protocol used in our study (Huot et al. 2001; Kalinichev et al. 2002; Park et al. 2011). Similar results were also observed in our MS rats. In contrast, the EOCO-treated MS rats showed less avoidance of the aversive open arms than the MS rats. For validating the effect of EOCO on anxiety-related behaviors in MS rats, we treated MS rats with fluoxetine. Fluoxetine, a serotonin selective reuptake inhibitor (SSRI), is a well-known antidepressant, and has been commonly used to treat mood and anxiety disorders. Previous studies have reported that treating MS rats (5 or 10 mg/kg) for 7 days (during pnd 2–9, 9–16, or 14–21) with fluoxetine alleviated depression-like behavior and the decrease in cell proliferation in the hippocampal dentate gyrus, as shown in MS rats (Lee et al. 2001; Freund et al. 2013). Baek et al. (2012) also showed that fluoxetine treatment (5 mg/kg) during pnd 21–30 protected MS rats from increased depression-like behavior. In our study, we treated MC and MS rats (pnd 21–28) with fluoxeine for 7 days, and then performed the EPM test. The frequency of open arm entries and the amount of time spent in open arms were increased in the fluoxetine-treated MS rats compared with the saline-treated MS rats, similar to the EOCO-treated MS rats. These results indicated that EOCO was similar to fluoxetine in decreasing anxiety-related behaviors in the MS rats, and thus, EOCO effectively alleviated the early life stress.

In our microarray analysis, EOCO upregulated or downregulated the expressions of 111 genes in the hippocampus of MS rats by more than 1.5-fold. When we performed the KEGG pathway analysis (http://www.genome.jp/kegg/pathway.html) to elucidate the biological functions of these genes, we found that these genes belonged to the following KEGG pathways (top 3 KEGG pathways in order of importance): cytokin–cytokine receptor interaction, chemokine signaling pathway, and herpes simplex infection. Of the 111 genes, a total of 14 genes belonged to the cytokin–cytokine receptor interaction (11 genes) and (or) chemokine signaling pathway (9 genes). Thus, we expected EOCO to affect cytokine genes in the hippocampus of MS rats. Indeed, we found that EOCO decreased the expressions of 10 cytokine genes, and increased the expressions of 4 cytokine genes in the hippocampus of MS rats. In particular, we found that the expressions of *Ccl2*, *Il6*, *Cxcl10*, *Ccl19*, and *Il1rl1* genes were upregulated in the MS group, and were significantly downregulated in the EOCO-treated MS group. The expression changes of these genes were also confirmed using RT–PCR.

Several studies have reported gene expression changes in the brains of MS rats or mice, using the microarray method. Benekareddy et al. (2010) reported that cellular developmental and neuronal plasticity-related genes such as neuroligin 1 (*Nlgn1*), glutamate receptor gene [glutamate receptor, ionotropic, *N*-methyl D-aspartate 2D (*Grin2d*)], and calcineurin genes [protein phosphatase 3 regulatory subunit B beta (*Ppp3r2*), Ppp3 catalytic subunit alpha isozyme (*Ppp3ca*)] were upregulated in the prefrontal cortex of MS Sprague–Dawley rats (pnd 2–14) separated from their mothers daily for 3 h. van Heerden et al. (2009) reported changes in the expression of genes involved in the glutamatergic and GABAergic systems in the prefrontal cortex and hippocampus of MS C57BL/6 mice (pnd 1–14) separated from their mothers daily for 3 h, providing evidence for the hyperglutamatergic state caused by MS. In the hippocampus of our MS rats, we also found that the expressions of glutamate receptor gene [glutamate receptor, ionotropic, AMPA 1 (*Gria1*), 1.650-fold] and calcineurin gene (*Ppp3r2*, 2.404-fold) were increased (data not shown). In addition, those studies showed changes in the cytokine genes. Expression of tumor necrosis factor receptor superfamily member 1b (*Tnfrsf1b*) was upregulated in the prefrontal cortex (Benekareddy et al. 2010), whereas expression of *Tnfrfs19* was downregulated in the hippocampus (van Heerden et al. 2009). Dimatelis et al. (2012) reported downregulation of cytokine genes in the whole brains of MS Wistar rats (pnd 2–14) separated from their mother daily for 3 h; they also showed decreased expressions of pro-inflammatory cytokines (*Il1b* and *Il5ra*), anti-inflammatory cytokine (*Il10*), and chemokines (*Ccl7* and *Ccr4*). In contrast, in our study, we found increases in 11 cytokine genes, including pro-inflammatory cytokines (*Il6*, *Il6ra* and *Ifng*), chemokines (*Ccl2*, *Cxcl10* and *Ccl19*), and *Tnfsf10* in the hippocampus of MS rats (see also supplementary data, Table S1[1]). Increased pro-inflammatory cytokine levels have been also shown in the plasma and brains of patients with depression as well as in MS animal models. Indeed, elevations in levels of CCL2, CCL3, IL1B, IL6, and TNF have been shown in the plasma of depressed patients (Simon et al. 2008; Janssen et al. 2010). In addition, increased expressions of cyclooxygenase 2 (Cox2) and Il1B have been shown in the prefrontal cortex of MS Sprague–Dawley rats (pnd 2–20) separated from their mothers daily for 4 h (Brenhouse and Andersen 2011), and in the hippocampus of MS piglets (pnd 3–11) separated from

[1]Supplementary data for this article are available with the article through the journal Web site at http://www.nrcresearchpress.com/doi/suppl/10.1139/cjpp-2013-0224).

Fig. 3. Effect of essential oil from *Chamaecyparis obtusa* (EOCO) on the protein expression of Ccl2 and Il6 in the hippocampus of maternal separation (MS) rats. Protein expressions of Ccl2 (A) and Il6 (B) were assessed in the hippocampus of the maternal care (MC), EOCO-treated MC, MS, and EOCO-treated MS rats using an enzyme-linked immunosorbent assay. Results are the mean ± SEM. Experiments were repeated 3 times using same samples; *, $p < 0.05$ for the MS group compared with the MC group; †, $p < 0.05$ for the EOCO-treated MS group compared with the MS group; one-way ANOVA followed by Tukey's post-hoc test.



their mothers daily for 2 h (Kanitz et al. 2004), respectively. Although not all data are consistent regarding the response of inflammatory cytokines to MS, this may be due to methodological factors (species type, MS protocol, brain region of cytokine determination), these findings suggests that MS may induce cytokine abnormality in the brain.

Interestingly, among 11 cytokine genes elevated by MS, EOCO reduced the expressions of 5 genes. In particular, we focused on the expressions of *Ccl2* and *Il6*, which were markedly decreased by EOCO in the MS rats. Thus, we examined whether EOCO also affected the protein levels of Ccl2 and Il6 in the rat hippocampus. The MS rats had increased levels of Ccl2 and Il6 compared with the MC rats, whereas the levels of Ccl2 and Il6 were decreased in the hippocampus of EOCO-treated MS rats compared to MS rats.

IL6 is one of the most widely studied cytokines in patients with depression. In addition to the elevation of this cytokine in depressed patients, IL6 concentration relates to the severity of depression (Prather et al. 2009; Hannestad et al. 2011), and a reduction in its concentration occurs in response to antidepressants (Basterzi et al. 2005). Moreover, an elevation of Il6 was found in the brains of MS Sprague–Dawley rats (pnd 2–14) separated from their mothers daily for 3 h (Desbonnet et al. 2010). Previous studies have also reported an increase of CCL2 in the plasma of patients with depression (Sutcigil et al. 2007; Piletz et al. 2009), and its reduction with antidepressant treatment (Sutcigil et al. 2007). In addition, a genetic association between *CCL2* and depression has been demonstrated (Altamura et al. 2010).

Although there have not yet been any reports to date showing that EOCO can modulate cytokines in MS animals or depressed patients, several studies have shown that EOCO affects cytokine modulation. Joo et al. (2010) reported that EOCO decreased mRNA expressions of *Il2*, *Infg*, and *Il5* in concanavalin A-activated mouse splenocytes. Shih et al. (2012) reported that a constituent of EOCO, β-thujaplicin, reduced Il6, Tnf, Cox2, and NF-κB protein expressions in lipopolysaccharide-stimulated macrophage cells (RAW 264.7). Thus, these reports and our results indicate that EOCO alleviates MS-induced cytokine abnormalities, and in particular Ccl2 and Il6, in the rat hippocampus.

In conclusion, EOCO protected against MS-induced anxiety-related behaviors and cytokine abnormalities. These results suggest that EOCO may be useful as a therapy for early life stress.

Acknowledgements

This study was carried out with the support of the Forest Science & Technology Projects (project No. S111113L020100) provided by Korea Forest Service.

References

Agid, O., Shapira, B., Zislin, J., Ritsner, M., Hanin, B., Murad, H., et al. 1999. Environment and vulnerability to major psychiatric illness: a case control study of early parental loss in major depression, bipolar disorder and schizophrenia. Mol. Psychiatry, **4**(2): 163–172. doi:10.1038/sj.mp.4000473. PMID: 10208448.

Altamura, A.C., Mundo, E., Cattaneo, E., Pozzoli, S., Dell'osso, B., Gennarelli, M., et al. 2010. The MCP-1 gene (*SCYA2*) and mood disorders: preliminary results of a case-control association study. Neuroimmunomodulation, **17**(2): 126–131. doi:10.1159/000258696. PMID:19923858.

Aoshima, H., and Hamamoto, K. 1999. Potentiation of $GABA_A$ receptors expressed in *Xenopus* oocytes by perfume and phytoncid. Biosci. Biotechnol. Biochem. **63**(4): 743–748. doi:10.1271/bbb.63.743. PMID:10361687.

Bae, D., Seol, H., Yoon, H.G., Na, J.R., Oh, K., Choi, C.Y., et al. 2012. Inhaled essential oil from *Chamaecyparis obtuse* ameliorates the impairments of cognitive function induced by injection of beta-amyloid in rats. Pharm. Biol. **50**(7): 900–910. doi:10.3109/13880209.2011.642886. PMID:22468783.

Baek, S.S., Jun, T.W., Kim, K.J., Shin, M.S., Kang, S.Y., and Kim, C.J. 2012. Effects of postnatal treadmill exercise on apoptotic neuronal cell death and cell proliferation of maternal-separated rat pups. Brain Dev. **34**(1): 45–56. doi:10.1016/j.braindev.2011.01.011. PMID:21353411.

Barnow, S., Lucht, M., and Freyberger, H.J. 2001. Influence of punishment, emotional rejection, child abuse, and broken home on aggression in adolescence: an examination of aggressive adolescents in Germany. Psychopathology, **34**(4): 167–173. doi:10.1159/000049302. PMID:11549925.

Basterzi, A.D., Aydemir, C., Kisa, C., Aksaray, S., Tuzer, V., Yazici, K., et al. 2005. IL-6 levels decrease with SSRI treatment in patients with major depression. Hum. Psychopharmacol. **20**(7): 473–476. doi:10.1002/hup.717. PMID:16158446.

Benekareddy, M., Goodfellow, N.M., Lambe, E.K., and Vaidya, V.A. 2010. Enhanced function of prefrontal serotonin 5-HT(2) receptors in a rat model of psychiatric vulnerability. J. Neurosci. **30**(36): 12138–12150. doi:10.1523/JNEUROSCI.3245-10.2010. PMID:20826676.

Brenhouse, H.C., and Andersen, S.L. 2011. Nonsteroidal anti-inflammatory treatment prevents delayed effects of early life stress in rats. Biol. Psychiatry, **70**(5): 434–440. doi:10.1016/j.biopsych.2011.05.006. PMID:21679927.

Desbonnet, L., Garrett, L., Clarke, G., Kiely, B., Cryan, J.F., and Dinan, T.G. 2010. Effects of the probiotic *Bifidobacterium infantis* in the maternal separation model of depression. Neuroscience, **170**(4): 1179–1188. doi:10.1016/j.neuroscience.2010.08.005. PMID:20696216.

Dimatelis, J.J., Pillay, N.S., Mutyaba, A.K., Russell, V.A., Daniels, W.M., and Stein, D.J. 2012. Early maternal separation leads to down-regulation of cytokine gene expression. Metab. Brain Dis. **27**(3): 393–397. doi:10.1007/s11011-012-9304-z. PMID:22527996.

Freund, N., Thompson, B.S., Denormandie, J., Vaccarro, K., and Andersen, S.L. 2013. Windows of vulnerability: Maternal separation, age, and fluoxetine on adolescent depressive-like behavior in rats. Neuroscience, **249**: 88–97. doi:10.1016/j.neuroscience.2013.06.066. PMID:23850503.

Hannestad, J., DellaGioia, N., and Bloch, M. 2011. The effect of antidepressant medication treatment on serum levels of inflammatory cytokines: a meta-analysis. Neuropsychopharmacology, **36**(12): 2452–2459. doi:10.1038/npp.2011.132. PMID:21796103.

Ho, C.L., Hua, K.F., Hsu, K.P., Wang, E.I., and Su, Y.C. 2012. Composition and antipathogenic activities of the twig essential oil of *Chamaecyparis formosensis* from Taiwan. Nat. Prod. Commun. **7**(7): 933–936. PMID:22908586.

Hong, E.J., Na, K.J., Choi, I.G., Choi, K.C., and Jeung, E.B. 2004. Antibacterial and antifungal effects of essential oils from coniferous trees. Biol. Pharm. Bull. **27**(6): 863–866. doi:10.1248/bpb.27.863. PMID:15187434.

Huot, R.L., Thrivikraman, K.V., Meaney, M.J., and Plotsky, P.M. 2001. Develop-

ment of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. Psychopharmacology (Berl.), **158**(4): 366–373. doi:10.1007/s002130100701. PMID:11797057.

Janssen, D.G., Caniato, R.N., Verster, J.C., and Baune, B.T. 2010. A psychoneuroimmunological review on cytokines involved in antidepressant treatment response. Hum. Psychopharmacol. **25**(3): 201–215. doi:10.1002/hup.1103. PMID:20373471.

Joo, S.S., Yoo, Y.M., Ko, S.H., Choi, W., Park, M.J., Kang, H.Y., et al. 2010. Effects of essential oil from *Chamaecypris obtusa* on the development of atopic dermatitis-like skin lesions and the suppression of Th cytokines. J. Dermatol. Sci. **60**(2): 122–125. doi:10.1016/j.jdermsci.2010.08.008. PMID:20869850.

Kalinichev, M., Easterling, K.W., Plotsky, P.M., and Holtzman, S.G. 2002. Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. Pharmacol. Biochem. Behav. **73**(1): 131–140. doi:10.1016/S0091-3057(02)00781-5. PMID:12076732.

Kanitz, E., Tuchscherer, M., Puppe, B., Tuchscherer, A., and Stabenow, B. 2004. Consequences of repeated early isolation in domestic piglets (*Sus scrofa*) on their behavioural, neuroendocrine, and immunological responses. Brain Behav. Immun. **18**(1): 35–45. doi:10.1016/S0889-1591(03)00085-0. PMID:14651945.

Kawakami, K., Kawamoto, M., Nomura, M., Otani, H., Nabika, T., and Gonda, T. 2004. Effects of phytoncides on blood pressure under restraint stress in SHRSP. Clin. Exp. Pharmacol. Physiol. **31**(Suppl. 2): S27–S28. doi:10.1111/j.1440-1681.2004.04102.x. PMID:15649280.

Lee, H.J., Kim, J.W., Yim, S.V., Kim, M.J., Kim, S.A., Kim, Y.J., et al. 2001. Fluoxetine enhances cell proliferation and prevents apoptosis in dentate gyrus of maternally separated rats. Mol. Psychiatry, **6**(6): 725–728. doi:10.1038/sj.mp.4000954. PMID:11673802.

Li, Q., and Kawada, T. 2011. Effect of forest environments on human natural killer (NK) activity. Int. J. Immunopathol. Pharmacol. **24**(1 Suppl.): 39S–44S. PMID:21329564.

Li, Q., Kobayashi, M., Wakayama, Y., Inagaki, H., Katsumata, M., Hirata, Y., et al. 2009. Effect of phytoncide from trees on human natural killer cell function. Int. J. Immunopathol. Pharmacol. **22**(4): 951–959. PMID:20074458.

Li, Q., Kobayashi, M., Inagaki, H., Hirata, Y., Li, Y.J., Hirata, K., et al. 2010. A day trip to a forest park increases human natural killer activity and the expression of anti-cancer proteins in male subjects. J. Biol. Regul. Homeost. Agents, **24**(2): 157–165. PMID:20487629.

Park, H.J., Chae, Y., Kim, J.W., Lee, H., and Chung, J.H. 2011. Effect of acupuncture on hypothalamic-pituitary-adrenal system in maternal separation rats. Cell. Mol. Neurobiol. **31**(8): 1123–1127. doi:10.1007/s10571-011-9718-x. PMID:21643998.

Piletz, J.E., Halaris, A., Iqbal, O., Hoppensteadt, D., Fareed, J., Zhu, H., et al. 2009. Pro-inflammatory biomakers in depression: treatment with venlafaxine. World J. Biol. Psychiatry, **10**(4): 313–323. doi:10.3109/15622970802573246. PMID:19921973.

Plotsky, P.M., and Meaney, M.J. 1993. Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Res. Mol. Brain Res. **18**(3): 195–200. doi:10.1016/0169-328X(93)90189-V. PMID:8497182.

Prather, A.A., Rabinovitz, M., Pollock, B.G., and Lotrich, F.E. 2009. Cytokine-induced depression during IFN-alpha treatment: the role of IL-6 and sleep quality. Brain Behav. Immun. **23**(8): 1109–1116. doi:10.1016/j.bbi.2009.07.001. PMID:19615438.

Pryce, C.R., Ruedi-Bettschen, D., Dettling, A.C., Weston, A., Russig, H., Ferger, B., et al. 2005. Long-term effects of early-life environmental manipulations in rodents and primates: Potential animal models in depression research. Neurosci. Biobehav. Rev. **29**(4–5): 649–674. doi:10.1016/j.neubiorev.2005.03.011. PMID:15925698.

Seol, G.H., Shim, H.S., Kim, P.J., Moon, H.K., Lee, K.H., Shim, I., et al. 2010. Antidepressant-like effect of *Salvia sclarea* is explained by modulation of dopamine activities in rats. J. Ethnopharmacol. **130**(1): 187–190. doi:10.1016/j.jep.2010.04.035. PMID:20441789.

Shih, M.F., Chen, L.Y., Tsai, P.J., and Cherng, J.Y. 2012. In vitro and in vivo therapeutics of beta-thujaplicin on LPS-induced inflammation in macrophages and septic shock in mice. Int. J. Immunopathol. Pharmacol. **25**(1): 39–48. PMID:22507316.

Simon, N.M., McNamara, K., Chow, C.W., Maser, R.S., Papakostas, G.I., Pollack, M.H., et al. 2008. A detailed examination of cytokine abnormalities in Major Depressive Disorder. Eur. Neuropsychopharmacol. **18**(3): 230–233. doi:10.1016/j.euroneuro.2007.06.004. PMID:17681762.

Sullivan, K.E., Mullen, C.A., Blaese, R.M., and Winkelstein, J.A. 1994. A multiinstitutional survey of the Wiskott–Aldrich syndrome. J. Pediatr. **125**(6): 876–885. doi:10.1016/S0022-3476(05)82002-5. PMID:7996359.

Sutcigil, L., Oktenli, C., Musabak, U., Bozkurt, A., Cansever, A., Uzun, O., et al. 2007. Pro- and anti-inflammatory cytokine balance in major depression: effect of sertraline therapy. Clin. Dev. Immunol. **2007**: 76396. doi:10.1155/2007/76396. PMID:18317531.

van Heerden, J.H., Conesa, A., Stein, D.J., Montaner, D., Russell, V., and Illing, N. 2009. Parallel changes in gene expression in peripheral blood mononuclear cells and the brain after maternal separation in the mouse. BMC Res. Notes, **2**: 195. doi:10.1186/1756-0500-2-195. PMID:19781058.

Veenema, A.H., Blume, A., Niederle, D., Buwalda, B., and Neumann, I.D. 2006. Effects of early life stress on adult male aggression and hypothalamic vasopressin and serotonin. Eur. J. Neurosci. **24**(6): 1711–1720. doi:10.1111/j.1460-9568.2006.05045.x. PMID:17004935.

Wilber, A.A., Southwood, C.J., and Wellman, C.L. 2009. Brief neonatal maternal separation alters extinction of conditioned fear and corticolimbic glucocorticoid and NMDA receptor expression in adult rats. Dev. Neurobiol. **69**(2–3): 73–87. doi:10.1002/dneu.20691. PMID:19025931.