Revisiting bupropion anti-inflammatory action: involvement of the TLR2/TLR4 and JAK2/STAT3

Alireza Karimollah[1], Anahid Hemmatpur[1], and Taha Vahid[1]

[1]Shahid Sadoughi University of Medical Sciences and Health Services

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Abstract

There are accumulating reports regarding poor response to the common antidepressant therapy. Antidepressant resistance has often been associated with activation of the inflammatory system. Accordingly, major depressive disorder (MDD) patients displaying inflammation prior to the treatment are less responsive to antidepressants. We hypothesized that the inefficacy of antidepressant therapy in some patients could be due to the drugs' inflammatory mode of action that remained overshadowed by their substantial therapeutic value. Bupropion is a common-used antidepressant that is prescribed for seasonal affective disorders and smoking cessation as well. Nevertheless, there are some reports regarding inflammation induction and depressive behavior exacerbation in response to bupropion. Here, we put a spot on bupropion and investigate the alterations of innate and adaptive immunity cytokines and the influence on immune signaling pathways. Therefore, we treated LPS-stimulated human peripheral mononuclear cells (PBMCs) with different doses of bupropion. Pro-/ anti-inflammatory cytokines (TNF-a, IL-1ß, IL-17, and IL-10) on both transcriptional and translational levels are assessed as well as the involvement of the JAK2 /STAT3, TLR2, and TLR4 signaling in this process. Bupropion decreased IL-17A, TNF-a, and IL-1ß protein levels in the cultures. Nonetheless, the results regarding the target genes expression were controversial. Surprisingly, IL-1ß, TNF-a, and IL-17A genes expression increased following bupropion treatment. TLR2, TLR4, JAK2, and STAT3 gene expression also rose in response to bupropion. Our findings suggest that bupropion possesses pro-inflammatory properties especially at concentrations of 50 and 100 and would rather be co-administrated with anti-inflammatory agents at least in patients with inflammatory conditions.

Revisiting bupropion anti-inflammatory action: involvement of the TLR2/TLR4 and JAK2/STAT3

Short title: Bupropion & inflammatory response

Alireza Karimollah [1]*, Anahid Hemmatpur[2], Taha Vahid [1]

[1]Department of Pharmacology, School of Pharmacy, Shahid Sadoughi University of Medical Sciences and Health Services, Shohaday-e-Gomnam Blvd., Alem Sq., Yazd, Iran

[2]Department of biochemistry, School of medicine, Shahid Sadoughi University of medical sciences and Health Services, Shohaday-e-Gomnam Blvd., Alem Sq., Yazd, Iran

*** Corresponding author**

Alireza Karimollah, PhD

E-mail address: karim2560@yahoo.com

Tel.: +98 (35) 3820 3410

Fax: +98 (35) 3725 4750

Principal investigator statement

The authors confirm that the PI for this paper is Alireza Karimollah and that he had direct clinical responsibility for patient.

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What is already known about this subject?

- A considerable proportion of depressed patients do not respond to the common antidepressant therapy.
- Inflammation is linked to the depression pathophysiology and inflammatory status prior to the treatment determines the success of medication in some MDD patients.
- Bupropion is a common-used antidepressant with the confirmed immunomodulatory properties.

What this study adds:

- This study revealed that despite the attributed anti-inflammatory properties to bupropion, it enhances gene expression of pro-inflammatory cytokines including IL-1ß, TNF-a, and IL-17A.
- Bupropion upregulates gene expression of TLR2, TLR4 and JAK2/STAT3 signaling molecules.
- Bupropion would better get administrated with anti-inflammatory agents.

ABSTRACT

There are accumulating reports regarding poor response to the common antidepressant therapy. Antidepressant resistance has often been associated with activation of the inflammatory system. Accordingly, major depressive disorder (MDD) patients displaying inflammation prior to the treatment are less responsive to antidepressants. We hypothesized that the inefficacy of antidepressant therapy in some patients could be due to the drugs' inflammatory mode of action that remained overshadowed by their substantial therapeutic value. Bupropion is a common-used antidepressant that is prescribed for seasonal affective disorders and smoking cessation as well. Nevertheless, there are some reports regarding inflammation induction and depressive behavior exacerbation in response to bupropion. Here, we put a spot on bupropion and investigate the alterations of innate and adaptive immunity cytokines and the influence on immune signaling pathways. Therefore, we treated LPS-stimulated human peripheral mononuclear cells (PBMCs) with different doses of bupropion. Pro-/ anti-inflammatory cytokines (TNF-a, IL-1ß, IL-17, and IL-10) on both transcriptional and translational levels are assessed as well as the involvement of the JAK2 /STAT3, TLR2, and TLR4 signaling in this process. Bupropion decreased IL-17A, TNF-a, and IL-1ß protein levels in the cultures. Nonetheless, the results regarding the target genes expression were controversial. Surprisingly, IL-1ß, TNF-a, and IL-17A genes expression increased following bupropion treatment. TLR2, TLR4, JAK2, and STAT3 gene expression also rose in response to bupropion. Our findings suggest that bupropion possesses pro-inflammatory properties especially at concentrations of 50 and 100 and would rather be co-administrated with anti-inflammatory agents at least in patients with inflammatory conditions.

Key words: Bupropion, response, TLR2, TLR4, JAK2/ STAT3

INTRODUCTION

The interactions between the immune and nervous systems are the major concern of psychoneuroimmunology. Current literature repeatedly highlighted the role of inflammation in depression pathogenesis and relapse [1,2]. Regarding, cytokine production pattern is indicated to be dysregulated in MDD patients with inflammatory etiology. Several meta-analyses reported elevated levels of TNF-a, IL6, and IL-1ß in the blood and brain of MDD patients [3,4]. IL-17 role in the pathophysiology of depression is also established[5]. Furthermore, mild stimulation of the immune system with lipopolysaccharides (LPS) could induce the symptoms of depression[6,7]. Therefore, the contribution of inflammatory signaling pathways to the pathophysiology of the disorder also took into consideration in recent documentations. In this regard, studies reported an imbalanced toll-like receptor (TLR)-mediated inflammatory response in MDD. TLR4 mRNA and protein levels were found to

be increased in both the periphery and CNS of MDD patients [4,8]. The Janus kinase/signal transducer and activator of transcription (JAK/STAT) inflammatory signaling pathway is also proposed to be pathologically linked to depression [9]. Regarding, evidence indicated that JAK2/STAT3 is crucial to neuroimmune system maintenance.

Antidepressants are believed to shift the immune balance toward anti-inflammatory responses. However, provocation of inflammatory responses (e.g. increased TNF-a and IL-6 levels) under treatment with serotonin and norepinephrine reuptake inhibitors (SNRIs) have been reported previously in both human and mouse models [4]. Despite the development of various classes of antidepressants, over 30% of patients don't achieve remission and 60% of remitted patients develop a new depressive episode [2,3]. Recent studies suggest that inflammatory status prior to the treatment determines the success of medication in MDD patients at least in the inflammatory form of the disease [1]. Moreover, MDD patients with multiple failed antidepressant treatments showed higher plasma levels of TNF and IL-6 [10]. Despite the existence of relatively large data discussing cytokine protein levels as immune system mediators in response to antidepressants, expression of correlated genes following antidepressant administration has remained disregarded.

During systemic inflammation, pro-inflammatory cytokines can pass through leaky parts of blood-brain barrier and induce neuroinflammation. Penetrating the brain, cytokines modulate the synthesis, release, and reuptake of neurotransmitters relevant to the mood including serotonin, norepinephrine, and dopamine [11]. Evidence implied that bupropion decreases IL-1ß, TNF-a, and IFN-γ levels while increases IL-10 levels [12]. Given that bupropion is a norepinephrine–dopamine reuptake inhibitor (NDRI) and a serotonin (5-HT) type 3A receptor inhibitor possessing anti-inflammatory properties [13], it is expected to have a double point advantage as an antidepressant. Meanwhile, in an animal study conducted by Helaly et al, bupropion exerted an inflammatory effect at therapeutic doses evidenced by positive NF-ϰB/ p65 stained microglia and neurocytes, obvious inflammation in hippocampus along with a moderate rise in the mean of serum IL- 6 levels [14]. Moreover, the association between bupropion administration and a couple of inflammatory conditions is stated in a considerable number of case reports [15-18]. Some investigations have also stated the possibility of bupropion-induced psychotic symptoms[19].

Based on the aforementioned observations, we hypothesize that the absence of the desired response to antidepressants, in our case bupropion, in some patients could be due to the not previously-noticed pro-inflammatory properties of the drug. To test the hypothesis, we evaluated the modulation of protein and gene expression of TNF-a, IL-1ß, IL-17, and IL-10 by bupropion. Gene expression of immunomodulatory signaling molecules including TLR2, TLR4, JAK2, and STAT3 was also assessed.

MATRIAL AND METHODS

Ethical statement

It is important to note that the volunteers gave consent to take part in the study. Tests with the use of human samples have been approved by University ethical committee with ethical code: ir.ssu.medicine.rec.1395.337.

PBMC isolation, culture, and treatment

Mutual interaction of microglia and immune cells is indicated to be present in depression. Therefore concerning microglia's central role in the development of depressive behavior, studies represented MDD as a microgliopathy [20]. Microglia are the present immune cells of the central nervous system and despite some differences, they share a similar progeny with peripheral immune cells. Given their similar progeny, microglia-like cells have been induced from the human peripheral blood monocyte cells in culture [21,22]. Therefore, PBMCs response could reflect the drug impact on both peripheral and microglia in patients as previously indicated[23]. Furthermore, according to the literature inflammatory status of the monocytes in MDD patients determines the response rates to some antidepressants [1]. Therefore, we chose the PBMCs model to explore bupropion immunomodulatory effects. PBMCs were isolated from the whole blood collected from 5 healthy volunteers via Ficoll (Baharafshan, Iran) gradient centrifugation and washed three times subsequently with RPMI-1640 medium (BioIdea, Iran). Cells were suspended in RPMI 1640 medium thereafter (supplemented with 100

U of penicillin/ml, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% Fetal Bovine Serum FBS) (FBS, Biochrom, Berlin, Germany). The cell viability was evaluated using trypan blue (Innoclon, Iran), and cells with a viability of more than 95% were selected for culture. Cells were transferred in duplicate into the wells of a 96-well microplate at the densities of 10^4 cells/well. The microculture plate was incubated at 37^{o}C under 5% CO2 with 90% humidity. To induce cell proliferation, phytohemagglutinin (10 µg/ml) (PHA, Sigma-Aldrich, USA) was added to the wells in the presence or absence of bupropion. LPS (100 ng/ml) was also added to the test culture and incubated at 37°C, 5% CO2, for 24 hours. Finally, bupropion was administered to PBMCs at different concentrations (25, 50, 100, 200 µM). Following 48-hour incubation, plates were centrifuged at 1700 RPM for 10 minutes, and Supernatants get collected.

Cytokine Array and Enzyme-Linked Immunosorbent Assay (ELISA)

After 72 h co-treatment of cells with PHA+ LPS and bupropion, the plate was centrifuged for 10 min, the supernatant removed, and get aliquoted. Cytokines concentration of IL-1β, IL-17, TNFα, and IL-10 were determined using ELISA kits (R&D Systems, US) according to the manufacturer's protocol. Optical density measurements took at 450 nm.

RNA isolation and RT-PCR

Total RNA isolated from 200 µL bupropion treated and untreated PBMCs using Total RNA Mini Kit (Yektatajhiz, Iran) and cDNA synthesized afterward using a cDNA synthesis kit (Yektatajhiz, Iran) according to the manufacturer's instruction. Relative mRNA expression of target genes including tumor necrosis factor-a (TNF-a), Interleukine1(IL-1β), Janus Kinase 2 (JAK2), and Signal transducer and activator of transcription 3 (STAT3) quantification conducted on Step One Plus real-time polymerase chain reaction (PCR) system (Applied Biosystems, ABI, Foster City, CA, USA) using SYBR Green qPCR Master Mix (Applied Biosystems, ABI, Foster City, CA, USA). β-actin mRNA levels used for results' normalization. Primer sequences are mentioned in Table 1.

Statistical Analysis

Statistical analysis performed using GraphPad Prism 6.07 Software. Data analyzed performing one way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. Data presented as mean ± Standard Deviation (SD). A P value of less than 0.05 was considered statistically significant.

RESULTS

Bupropion effect on concentration of target cytokines in PBMCs culture

To discover the alterations in pro-inflammatory cytokines profile in response to bupropion, LPS-PHA treated peripheral blood mononuclear cells get treated with different concentrations of bupropion. As depicted in graphs (Fig. 1A-C), bupropion administration significantly decreased the secretion of pro-inflammatory cytokines including TNF-a, IL-1ß, and IL-17 levels in media assessed by ELISA. We further evaluated the levels of anti-inflammatory cytokine IL-10; bupropion treatment resulted in a significant increase in IL-10 concentration in the test cell culture (Fig.1D).

Bupropion effect on gene expression of target cytokines in PBMCs culture

In search of the inflammatory response to bupropion treatment at the gene expression level, we assessed the relative expression of pro-inflammatory interleukins performing real-time PCR. Interestingly, mRNA levels of pro-inflammatory cytokines including TNF-a, IL-1ß, and IL-17 were upregulated in cultures following bupropion incubation (Fig. 2A-C). Bupropion treatment also increased mRNA levels of IL-10 compared to the untreated culture (Fig. 2D).

Bupropion effect on the gene expression of TLR2 and TLR4

TLRs are key components of innate immunity and orchestrate immune responses by inducing the production of many cytokines. Concerning the recognized role of TLR2, TLR4 in depression, and as the representative

of innate and adaptive immunity we focused on TLR2, TLR4 signaling pathways. Bupropion addition to LPS-treated PBMCs significantly increased gene expression of both receptors (Fig. 3).

Tropisetron effect on the gene expression of JAK2 and STAT3

Several cytokines signal through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway which is involved in the development and modulation of immune responses. Among the human JAK four family members, JAK2 is the most common kinase activated by two-thirds of ligands. Following activation, STAT3 can promote the production of both inflammatory cytokines like IL-17 and IL-6 and anti-inflammatory cytokines (like TGF-β and IL-10) [24]. We focused on this kinase and related transcription factor to elucidate the molecular signaling pathway that might account for the bupropion immunomodulatory actions. Bupropion treatment led to a considerable rise in gene expression of JAK2 at 50 and 100 concentrations (Fig. 4A). We also found out that LPS-treated PBMCs significantly enhanced STAT3 gene expression in the presence of bupropion (Fig. 4B).

Dose-related effects of bupropion

Based on our findings, bupropion immunomodulatory effect varied depending on the concentration. Results demonstrated that bupropion exerts the most inflammatory action in 50 and 100 μM evidenced by a sharp increase in mRNA levels of target pro-inflammatory cytokines (TNF-a, IL-1ß, and IL-17) and considerable upregulation of TLR2, TLR4, JAK2, and STAT3 genes.

DISCUSSION

Bupropion is a common antidepressant that has been prescribed for over 20 years as a sole antidepressant or with other classes of antidepressants. Meanwhile, literature has reported the low response rates to bupropion in the patients with treatment-refractory depression[25]. Low-grade inflammation is believed to be involved in the poor responsiveness to regular antidepressants[1]. Moreover, despite the repeatedly mentioned anti-inflammatory effects, some controversies are reported regarding bupropion action including the development of some inflammatory abnormalities such as type 1 hypersensitivity reactions (erythema multiformeis) and serum sickness-like reaction [26,27]. Moreover, Eller et al observed elevated levels of IL-8 in response to bupropion [28]. Bupropion administration also failed to lower interleukins secretion in monocyte-derived macrophages collected from HIV positive women [29]. Therefore, we sought to determine bupropion inflammatory effects and underlying pathways in LPS-stimulated PBMCs. Present results revealed that bupropion might induce inflammatory responses via upregulation of TLR2, TLR4, JAK2, and STAT3 and consequently the pro-inflammatory cytokines gene expression.

In consistence with previous reports [30,31], bupropion inhibited secretion of TNF-a, IL-1-ß by LPS-stimulated PBMCs. Data addressing transcriptional changes in cytokine genes following bupropion administration is almost scarce. Surprisingly, bupropion increased mRNA levels of TNF-a, IL-1-ß, in the present study. However, in an animal study conducted by Helaly et al TNF-a gene expression reported being down-regulated by bupropion [14]. Studies concerning changes in IL-17 levels in response to bupropion are limited. In 2013 Kim et al failed to find a significant change in IL-17 levels under bupropion treatment [32]. However, an interesting study indicated that a higher baseline IL-17 level is associated with a greater reduction in depression severity in a bupropion-SSRI treatment arm [5]. Assessing IL-17A expression at both translational and transcriptional levels, we figured out that bupropion significantly increased the cytokine gene expression whereas it somehow inhibited the protein levels. Th17 cells and its major cytokine product, IL-17A, promote activation of microglia and astrocytes that may contribute to the neuroinflammation, neuronal damage, and consequently lead to depression. In addition to the Th17 cells, CD8+ cells, invariant natural killer T cells, monocytes, neutrophils, and γδ T cells also secrete IL-17A. IL-17A facilitates monocyte penetration through the blood-brain barrier, enhances the blood-brain barrier permeability, and extends the damage by attracting cytokine-producing cells[33]. Reaching the brain, IL-17A can activate microglia via present IL-17 receptors and indirectly induce the release of cytokines and chemokines that exacerbates the inflammation.

The abnormally high level of pro-inflammatory cytokines in MDD reflects the overactive TLR-mediated

signaling. Evidence indicated that both protein and mRNA expression of TLRs including TLR2 and TLR4 are higher in the central nervous system and peripheral blood of depressed patients. Bupropion capability to attenuate the deregulated expression of TLR4 and TLR2 has been suggested by few studies[34,35]. However, our obtained results concerning TLR4 expression profile differed depending on the drug concentration. Bupropion inhibited TLR4 expression at 25 and 200 μM, though it enhanced TLR4 expression at 100 μM. Bupropion also increased TLR2 gene expression in stimulated PBMCs. Microglia, the immune cells of the brain, also express TLR2 and TLR4 and provide pro-/anti-inflammatory cytokines as mediators for the neuroimmune system. Moreover, microglia showed an enhanced response to cytokines in MDD patients. Inflammation-induced by the activation of TLRs in microglia plays a substantial part in the pathology of depression [36,37]. Furthermore, TLR4 mRNA and protein reported being higher in both the periphery and CNS of MDD patients [4]. J. Hines et al demonstrated that intraperitoneal injection of Tat-TLR4 interfering peptides improves LPS-induced sickness behavior, an important contributor to the occurrence of depressive-like behavior, via rescuing the microglia morphology changes and cytokine production that are normally induced by LPS [38]. More importantly, negative regulators of the TLR signaling pathway have been suggested as potential predictive biomarkers of response to antidepressant treatment.

In search of signaling pathways underlying bupropion immunomodulatory action, Tsai et al reported that bupropion did not affect LPS-induced phospho-p65 and phospho-JNK, and phospho-ERK and phospho-p38 expression in THP-1 cells [7]. Besides the well-known role in the peripheral immune responses, a growing body of evidence proposed that the JAK2-3 signaling pathway is associated with neuroinflammation. JAK2-3 signaling and consequent secretion of TNF-α and IL-1β by microglia promote neuronal death and can consequently result in anxiety and depression [11,39]. JAK2 and 3 genes stated being over-expressed in both MDD patients and mice model with inflammation- induced depressive behavior [40,41]. Blocking JAK2-3 signaling inhibited pro-inflammatory cytokines release and attenuated neuroinflammation caused by activated microglia [42]. Moreover, intrathecal injection of a JAK2-3 cascade inhibitor considerably normalized the spinal inflammatory profile caused by peripheral inflammation [43,44]. JAK2-STAT3inhibitors have been proposed as a beneficial strategy in the treatment of depression [9,45,46]. Therefore, we investigated the gene expression of JAK2 and 3 in LPS-treated PBMCs after bupropion treatment. JAK2 expression was upregulated at 50 and 100 μM concentrations of the drug. 3 expression was also increased considerably at all concentrations of bupropion.

Taken together bupropion might contribute to peripheral and CNS inflammation via modulating TLR2/TLR4 and JAK2/STAT3 signaling pathways, particularly at moderate concentrations. We concluded that lack of expected response to bupropion in MDD patients with inflammatory etiology could be due to this unnoticed mode of action. Therefore, bupropion would better get administrated with anti-inflammatory agents. In confirmation of our results, studies demonstrated that bupropion combination with anti-inflammatory agents yielded a better outcome[47,48]. Of note, bupropion is an antidepressant with complex mechanisms of action including the recently discovered effect on the serotonin receptors. Dopamine, norepinephrine, acetylcholine, and serotonin each exert a specific immunomodulatory effect. Therefore, more comprehensive studies should get conducted to understand the net immunomodulatory effect of bupropion under both inflammatory and immunologically-balanced status in patients with depressive-like behavior. Our findings also notify the importance of a personalized medication approach in MDD patients regarding bupropion prescription.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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DATA AVALABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. qPCR primer sequences

Primer	Forward	Reverse
-actin β	**5'- CTGGAACGGTGAAGGTGACA -3'**	**5'- AAGGGACTTCCTGTAACAATGCA -3'**
TLR2	**5'- GGGTTGAAGCACTGGACAAT -3'**	**5'- TTCTTCCTTGGAGAGGCTGA -3'**
TLR4	**5'- TGGAAGTTGAACGAATGGAATGTG -3'**	**5'- ACCAGAACTGCTACAACAGATACT -3'**
JAK2	**5'- CCGATCTGTGTAGCCGGTTT -3'**	**5'- GTAAGGCAGGCCATTCCCAT -3'**
STAT3	**5'- TCCTGAAGCTGACCCAGGTA -3**	**5'- TCCTCACATGGGGGAGGTAG -3**

Primer	Forward	Reverse
IL-1-ß	**5'- CAGAAGTACCTGAGCTCGCC -3**	**5'- AGATTCGTAGCTGGATGCCG -3**
TNF-a	**5'- CCGATGGGTTGTACCTTGTC -3**	**5'- GTGGGTGAGGAGCACGTAGT -3**
IL-17	**5'- TGGAGGCCATAGTGAAGG -3**	**5'- TAGTGCTGAGGAGATGTTGC -3**
IL-10	**5'- ACCTCGACTCGCCTACAAAG -3**	**5'- GGCCACATGGTGGACAAT -3**

FIGURE LEGENDS

Figure 1. Bupropion effect on the secretion of the pro-/anti-inflammatory cytokines. Bupropion treatment significantly suppressed (A) IL-1ß, (B) TNF-a, and (C) IL-17 concentration while increased the (D) IL-10 levels in PBMCs media. The results are presented as mean ± S.D, * $p < 0.05$, *** $p < 0.001$, and **** $P < 0.0001$. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Sidak's multiple comparisons test.

Figure 2. Bupropion effect on gene expression of target cytokines gene. Bupropion significantly enhanced the gene transcription of (A) IL-1ss, (B) TNF-a, (C) IL-17, and (D) IL-10. Fold changes are calculated with the $2^{-[?][?]Ct}$ method. The results are presented as mean ± S.D, * $p < 0.05$, ***$p < 0.001$, and **** $P < 0.0001$. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Sidak's multiple comparisons test.

Figure 3. Bupropion effect on gene expression of TLRs. Treatment of LPS+PHA-stimulated cells with bupropion promoted the mRNA expression of (A) TLR2 and (B) TLR4. Fold changes are calculated with the $2^{-[?][?]Ct}$ method. The results are presented as mean +- S.D, * $p < 0.05$, ***$p < 0.001$, and **** $P < 0.0001$. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Sidak's multiple comparisons test.

Figure 4. Bupropion effect on gene expression of JAK2 /STAT3. Treatment of LPS+PHA-stimulated cells with bupropion promoted the mRNA levels of (A) JAK2 and (B) STAT3. Fold changes are calculated with the $2^{-[?][?]Ct}$ method. The results are presented as mean +- S.D, * $p < 0.05$, ***$p < 0.001$, and **** $P < 0.0001$. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Sidak's multiple comparisons test.