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Effect of Opium and Related Detoxification Drugs on RAW 264.7 Macrophages

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Abstract

Introduction: Detoxification drugs used for opium (OPM) withdrawal, such as buprenorphine (BUP), naltrexone (NTX), and methadone (MTD), can have inhibitory effects. In a recent study, the impact of OPM and these detoxification drugs on cytokines secreted by RAW 264.7 macrophages was examined.

Methods: The M1 model (positive control) included lipopolysaccharide (LPS)-stimulated macrophages, while the M0 model (negative control) included DMSO-treated macrophages. The study measured the ratios of inflammatory (IFN-γ, TNF-α, IL-6) to anti-inflammatory cytokines (IL-10, TGF-β) secreted by RAW 264.7 macrophages exposed to OPM and the detoxification drugs using real-time polymerase chain reaction (PCR).

Results: The results showed that in the M1 model, the ratio of IFN-γ/IL-10 significantly increased with OPM+MTD ($P<0.001$) and OPM+BUP ($P<0.05$). Similarly, the INF-γ/TGF-β ratio increased significantly with OPM+MTD ($P<0.001$), OPM+BUP ($P<0.01$), and OPM+NTX ($P<0.05$) compared to the negative control. Compared to the M0 model, the TNF-α/IL-10 and TNF-α/TGF-β ratios showed the highest increase with OPM+BUP ($P<0.001$) and OPM+MTD ($P<0.01$). Additionally, OPM+BUP ($P<0.001$) and OPM+MTD ($P<0.05$) exhibited the greatest increase in the IL-6/IL-10 ratio, and OPM+BUP ($P<0.001$) also showed a significant upward trend in the IL-6/TGF-β ratio.

Conclusion: These findings suggest that BUP and MTD may have the potential to be used as therapies for reducing inflammation in OPM-treated macrophages by increasing M1 cytokines and enhancing immune responses.

Keywords: Opium, Macrophage, Inflammation, Buprenorphine, Naltrexone, Methadone

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Introduction

Opioid addiction is a severe and growing problem affecting many people at different levels. Currently, many international communities dedicate a large part of their budget to engaging in the fight against this issue.[1] Opioids are natural and synthetic compounds with morphine-like effects affecting the central nervous system and immune systems through interaction with their receptors.[2] Depending on the origin and production process, these substances are divided into natural, semi-synthetic, synthetic, endogenous, and antagonistic products.[3,4] Among natural drugs, opium (OPM) with a high frequency of consumption can be mentioned. It is a natural narcotic drug that is extracted from the unripe seedpods of the OPM poppy (Papaver somniferum), a plant of the family Papaveraceae, and is widely used in the Middle East.[5-7]

Morphine is one of the most potent alkaloids in OPM.[8] In recent years, human and animal studies have shown that drugs such as morphine can increase susceptibility to chronic infections by reducing the inflammatory phase and adverse effects on the innate and adaptive immune cells.[9,10] Morphine can affect the immune system directly by binding to Mu (μ) opioid receptors (MORs) present on leukocytes or indirectly by acting on the central nervous system.[11,12] An opioid withdrawal program, known as therapeutic remission, uses narcotic-like substance (agonists) or narcotic antagonist.[13,14] Among these detoxification drugs, methadone (MTD), naltrexone (NTX), and buprenorphine (BUP) act as agonist, antagonist, and semi-agonist on different types of opioid receptors expressed on immune cells, respectively.[15-17]

Macrophages, involved in inflammation, are among the primary immune cells affected by opioids due to their opioid receptors.[18] Macrophages respond quickly to various stimuli, including bacterial, viral, fungal, and parasitic infections and tissue damage, and can acquire distinct functional phenotypes depending on their microenvironment.[19] Macrophages could be categorized into various phenotypes depending on the type of stimulus they are exposed to. As the macrophages are stimulated with lipopolysaccharide (LPS) and interferon-gamma (IFN-γ), they are polarized toward M1 macrophages, in which they can have inflammatory activity and play a role in host defense against external pathogens.[20] On the other hand, the M2 phenotype is differentiated by IL-4 or IL-13, which has anti-inflammatory functions.[21] M1 macrophages produce mediators associated with increased phagocytic activity and inflammation, such as induction of TNF-α, IL-6, and INF-γ. In contrast, M2 macrophages exhibit homeostatic and anti-inflammatory functions and secrete IL-10, IL-4, and TGF-β.[22,23]

Although the number of studies focused on the effects of OPM and its detoxification drugs is increasing in vitro and in vivo, our knowledge of the immunomodulatory effects of these substances and their related detoxification drugs is still insufficient. To date, no comparison has been made regarding the efficacy of using OPM separately and in combination with MTD, BUP, and NTX in modulating immune responses through the macrophage polarization process.

Materials and Methods

Cell Line, Chemicals, and Reagent

RAW 264.7 mouse macrophage cells were obtained from the Pasteur Institute (Tehran, Iran). The RNA Extraction Kit and the cDNA Synthesis Kit were purchased from Yekta Tajhiz (Yekta Tajhiz Azma, Iran). DMEM medium, fetal bovine serum (FBS), penicillin, streptomycin, dimethyl sulfoxide (DMSO), LPS, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were provided by Sigma-Aldrich (Sigma, USA). OPM was provided by Darou Pakhsh Pharmaceutical Manufacturing Company in Tehran, Iran. MTD, BUP, and NTX were obtained from Darou Pakhsh (Tehran, Iran), Mehr Darou (Tehran, Iran), and Iran Darou (Tehran, Iran) pharmaceutical companies, respectively.

Cell Culture and Treatments

RAW264.7 is a murine macrophage-like cell line that is used to evaluate M1 and M2 cytokine expression. RAW264.7 cells were cultured in complete DMEM, supplemented with 10% FBS, 100 U/mL of penicillin, and 100 U/mL of streptomycin. All cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Finally, 100 μg/mL LPS was used as an activator of M1 RAW264.7 macrophages, and DMSO-treated RAW264.7 cells were described as M0 macrophages.

IC50 Calculation

RAW264.7 cells were seeded in a 96-well microplate (SPL Life Sciences, Korea) containing complete DMEM medium for maximum concentration with the minimum toxicity measurement. The drugs were dissolved in the appropriate solvent (DMSO). RAW264.7 cells treated only with DMSO as the solvent were considered as negative control or M0 macrophages. RAW264.7 cells treated with 100 μg/mL LPS were considered as positive control or M1 macrophages. RAW264.7 cells were treated with different concentrations of OPM (18.75, 37.5, 75, 150, 300, 600 μmol/L), MTD (7.5, 15, 30, 60, 120, 240 μmol/L), BUP (0.75, 1.5, 3, 6, 12, 24 μmol/L), and NTX (0.75, 1.5, 3, 6, 12, 24 μmol/L). After 36 hours, 10 μL/well of MTT (5 mg/mL) was added to each well, and the cells were incubated for 4 hours. Formazan crystals were dissolved by adding 100 μL DMSO and measured by an ELISA reader at 570 nm. The half-maximal inhibitory concentration (IC50) was calculated by Excel using the linear trend line formula. The viability of the treated cells was calculated according to the following formula:

$$\text{Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

Relative Quantitative Real-time PCR

Total cellular RNA was extracted from treated RAW264.7 cells, and cDNA was synthesized according to the manufacturer's protocol (Yekta Tajhiz, Iran). Forward and reverse primers of pro-inflammatory cytokines, including IL-6, TNF-α, and INF-γ as M1 cytokines and anti-inflammatory cytokines like TGF-β and IL-10 as M2 cytokines, and GAPDH as housekeeping gene were designed using AlleleID 6.0 (Premier Biosoft International, USA) (Table 1). LPS-induced M1 macrophages were used as a positive control. The gene expression was evaluated by a real-time PCR assay run in triplicate using SYBR Green I and 2X Master Mix (Yekta Tajhiz, Iran) in a LightCycler 96 System (Roche, Switzerland). The melting curve analyzed the PCR products to confirm the lack of non-specific products. Additionally, relative quantification of target genes was performed using the Pfaffle method. The IL-6/IL-10, IL-6/TGF-β, TNF-α/IL-10, TNF-α/TGF-β, INF-γ/IL-10, and INF-γ/IL-10 ratios were calculated to determine M1/M2 macrophage gene expression.

Statistical Analysis

Statistical analyses were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). Kolmogorov–Smirnov test was used to evaluate the assumption of normality. ANOVA and Tukey's post hoc tests were also used to evaluate differences in variables between the studied groups. A *P*value less than 0.05 was considered statistically significant. Data were expressed as mean ± standard error

of the mean (SEM).

Results

Determination of IC50

The results of the MTT assay showed that the IC50 values of OPM, MTD, BUP, and NTX on treated RAW264.7 cells were 150, 60, 3, and 16 µmol/L, respectively.

Table 1. PCR Primer Sequences

Gene	Primers	Sequences 5'→ 3'
GAPDH	Forward	CGGTGTGAACGGATTTGG
	Reverse	CTCGCTCCTGGAAGATGG
IL-6	Forward	GAAATGATGGATGCTACCAAACTG
	Reverse	TCTGTATCTCTCTGAAGGACTCTG
TNF-α	Forward	CAGACCCTCACACTCACAAACC
	Reverse	GAAGAGAACCTGGGAGTAGACAAG
INF-γ	Forward	AGGAACTGGCAAAAGGATGG
	Reverse	GACCTCAAACTTGGCAATACTC
IL-10	Forward	CACTGCTATGCTGCCTGCTC
	Reverse	ACCCAAGTAACCCTTAAAGTCCTG
TGF-β	Forward	AATTCCTGGCGTTACCTTGG
	Reverse	GGCTGATCCCGTTGATTTCC

The Ratio of INF-γ to IL-10 and TGF-β Cytokines

As shown in Figure 1A, the IFN-γ/IL-10 ratio in RAW264.7 cells exposed to OPM + MTD ($P < 0.001$) and OPM + BUP ($P < 0.05$) was increased compared to the negative control (M0 macrophages). Moreover, in the INF-γ/TGF-β ratio (Figure 1B), an increase compared to negative control was observed in OPM + MTD ($P < 0.001$), OPM + BUP ($P < 0.01$), and OPM + NTX ($P < 0.05$).

The Ratio of TNF-α to IL-10 and TGF-β Cytokines

Other Cytokine ratios selected to determine the M1/M2 balance were TNF-α/IL-10 and TNF-α/TGF-β. As shown in Figures 2A and 2B, a significant increase compared to the control group (M0 Model) was observed in OPM + BUP ($P < 0.001$) and OPM + MTD ($P < 0.01$).

The Ratio of IL-6 to IL-10 and TGF-β Cytokines

As shown in Figure 3A, OPM + BUP ($P < 0.001$) and OPM + MTD ($P < 0.05$) had the highest increases in the

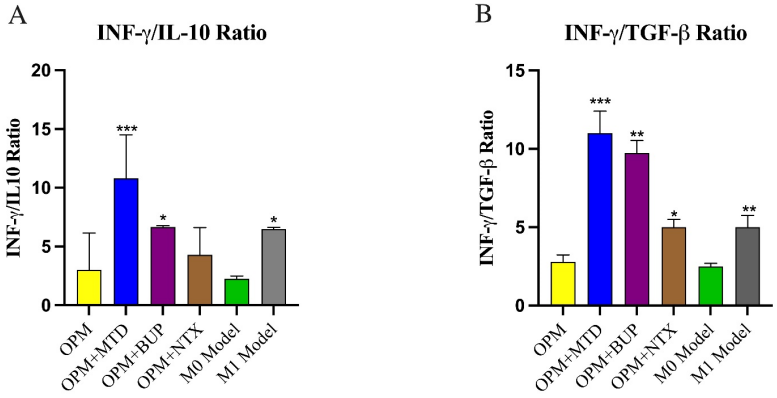

Figure 1. The Ratio of mRNA Expression of IFN-γ to Anti-inflammatory cytokine in RAW264.7 cells treated with OPM and detoxification drugs. (A) The ratio of IFN-γ/IL-10 in RAW264.7 cells exposed to OPM and its detoxification drugs; (B). The ratio of IFN-γ/TGF-β in RAW264.7 cells exposed to OPM and its detoxification drugs. Values are presented as mean ± standard error of mean vs. control group. OPM: Opium, MTD: Methadone, BUP: Buprenorphine, NTX: Naltrexone, M0 Model: Dimethyl sulfoxide treated macrophages, M1 Model: Lipopolysaccharides-induced macrophages. Values are presented as mean ± standard error of mean vs. control group (M0 model) (*$P < 0.05$) (**$P < 0.01$) (***$P < 0.001$)

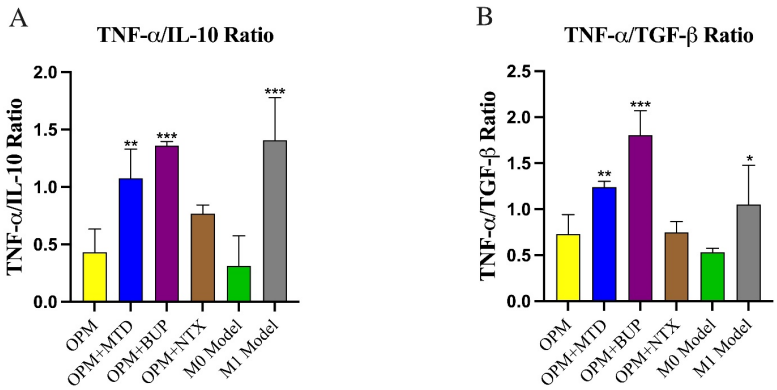

Figure 2. Treatment of RAW264.7 macrophages with detoxification drugs at indicated concentrations enhances the ratio of TNF-α to anti-inflammatory cytokines. **(A)** TNF-α/IL-10 ratio in RAW264.7 cells exposed to opium and its detoxification drugs; (B). TNF-α/TGF-β ratio in RAW264.7 cells exposed to opium and its detoxification drugs. Values are presented as mean ± standard error of mean vs. control group. (*$P < 0.05$). OPM: Opium, MTD: Methadone, BUP: Buprenorphine, NTX: Naltrexone, M0 Model: Dimethyl sulfoxide treated macrophages, M1 Model: Lipopolysaccharides-induced macrophages. Values are presented as mean ± standard error of mean vs. control group (**$P < 0.01$) (***$P < 0.001$)

IL-6/IL10 ratio. There was also a significant increase in the IL-6/TGF-β ratio in OPM + BUP ($P < 0.001$) (Figure 3B). The greatest dissimilarity between the experimental group and the positive control (M1 Model) was observed in OPM + MTD and OPM + BUP in both ratios.

Discussion

Opium is one of the most frequently used drugs in the Middle East and South Asia.[24] Many studies on people with opioid use disorders show that their immune systems are severely suppressed, including significant cellular immunodeficiency and T-cell genetic damage.[25] On the other hand, treating such individuals with withdrawal drugs like BUP, MTD, and NTX may also amplify the immune responses. For instance, with long-term MTD treatment, suppression of cellular immunity in patients with drug use disorder can switch to the normal condition.[25] Accordingly, in a clinical trial on MTD and BUP, the weakened immune system was activated in people who used heroin.[26]

Several investigations have demonstrated that OPM and many natural-based drugs reduce inflammatory factors, including cytokines related to macrophages, which are essential effector cells in inflammatory conditions.[27,28] This study evaluated the effect of OPM and related detoxification drugs on the RAW264.7 cell line and its cytokine expression to determine the polarization of macrophages and M1/M2 balance. Initially, the highest concentration with the lowest cell cytotoxicity was determined for OPM and its detoxification drugs, including MTD, BUP, and NTX.

After determining the appropriate concentration of these compounds by MTT assay, their immunomodulating effects on RAW264.7 gene expression were evaluated by the ratio of INF-γ to anti-inflammatory cytokines (IL-10 and TGF-β). OPM + MTD and OPM + BUP significantly increased the INF-γ/IL-10 ratio compared to M0 Model. The results also indicate an almost similar pattern in OPM + BUP, OPM + MTD, and OPM + NTX, which significantly upregulated the INF-γ/TGF-β ratio. These studies demonstrated that treatment with OPM and its detoxification drugs, unlike treatment with only OPM, increases the ratio of M1/M2-related cytokines and also triggers the RAW264.7 cells to reach the level of the M1 model cytokine that can boost the impaired inflammatory phase of macrophages. The highest increase in both ratios was seen in the OPM + MTD. These findings were consistent with previous studies that assessed the production of inflammatory cytokines in a group of heroin-addicted patients treated with MTD.

The results showed that IL-1β, IL -6, and IL-8 levels in the MTD treatment group were significantly higher than in the healthy control group. Increased plasma levels of TNF-α and IL-6 were significantly associated with MTD dose, and IL-1β levels were significantly correlated with the duration of MTD treatment.[29] In another study on cytokine release of the peritoneal macrophages obtained from inbred CBA mice treated with morphine, oxycodone, and BUP for 7 days, IL-6 concentration in the oxycodone and BUP treated groups was increased compared to morphine without LPS stimulation after 24 hours of culture.[30] In another study, myeloid DCs (CD11c +) and plasmacytoid DCs (CD123 +), subsets of monocytes like macrophages, were examined in chronic heroin addicts before and after detoxification with MTD. Considering the essential role of dendritic cells in initiating primary immune responses and inflammatory mediators, expression levels of CD11c and CD123 were normalized and increased in the chronic heroin addicts after MTD treatment.[31]

Other ratios that were studied to determine changes in M2 and M1-related cytokines were the ratio of TNF-α to IL-10 and TGF-β. A profound increase in TNF-α to IL-10 and TGF-β ratios was observed in treatment groups in which OPM was combined with BUP and MTD. Among all treatment groups, OPM + BUP represented the highest increase in the mentioned ratios. Other results of

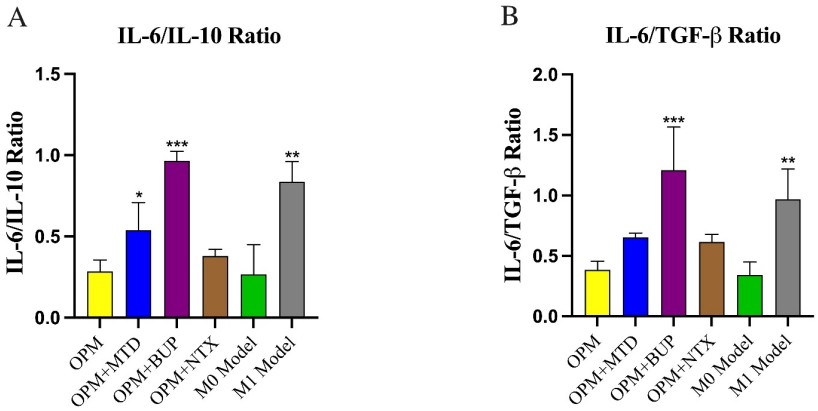

Figure 3. Evaluation of IL-6 to Anti-inflammatory Cytokine Ratios in RAW264.7 Macrophages Treated With Detoxification Drugs at Desired Concentrations. (A) The ratio of IL-6/IL-10 in RAW264.7 cells exposed to opium and its detoxification drugs; (B) Ratio of IL-6/TGF-β in RAW264.7 cells exposed to opium and its detoxification drugs. Values are presented as mean ± standard error of mean vs. control group (*$P < 0.05$). OPM: Opium, MTD: Methadone, BUP: Buprenorphine, NTX: Naltrexone, M0 Model: Dimethyl sulfoxide treated macrophages, M1 Model: Lipopolysaccharides-induced macrophages. Values are presented as mean ± standard error of mean vs. control group (*$P < 0.05$) (***$P < 0.001$)

this study were the immunomodulating effect of these compounds on IL-6 (M1-related cytokine), IL-10, and TGF-β (M2-related macrophage). Similar to the previous ratio assessment, the highest increase was observed in the group of OPM + BUP. Based on previous experiments, in a study of morphine and BUP-injected mice, peritoneal markers of the macrophages, such as MHC class II, CD80, and CD86, as well as markers of M1 macrophages, were downregulated in morphine-treated mice compared with the control group. However, the percentage of macrophages with these markers was higher in BUP-treated cells.[30] The limitations of this study were the lack of in vivo evaluation and assessment of other markers of M1 and M2 macrophages.

Conclusion

The macrophage is one of the central cells in the immune system and has a receptor for OPM. This experiment showed that detoxification drugs could switch the gene expression of RAW264.7 cells toward the M1 phenotype. Among these drugs, the highest increase in the ratio of M1/M2 cytokines was observed in BUP and MTD, which act as agonist and semi-agonist on different types of opioid receptors of the immune cells, respectively. Our results suggest that BUP and MTD may be promising therapies for induced inflammation in OPM-treated macrophages owing to the increase of M1/M2 cytokines and reaching the M1 model cytokine ratios.

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Competing Interests

The authors report there are no competing interests to declare.

Ethical Approval

This study was approved by the Ethics Committee of Arak University of Medical Sciences (IR.ARAKMU.REC.1399.266).

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