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The plasma levels of the cytokines in opium-addicts and the effects of opium on the cytokines secretion by their lymphocytes

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ABSTRACT

The aim of this study was to evaluate the effects of opium addiction on the secretion of IL-4, IFN-γ, IL-6 and TGF-β under *in vivo* and *in vitro* conditions. The blood samples were collected and PBMCs were cultured in RPMI1640 with and without opium for 48 h. The levels of the cytokines were measured using ELISA technique. The results showed that plasma levels of IL-4 and IFN-γ were significantly lower and IL-6 and TGF-β were higher in plasma taken from opium-addicted subjects. The concentrations of all the cytokines in opium-addicted subjects in *in vitro* condition were significantly lower than the control group. Addicted subjects cultured lymphocytes significantly decrease secreted IL-4, IL-6 and TGF-β but not IFN-γ in response to being cultured with opium, where as IFN-γ was increased in controls. These results may explain the frequent microbial infections and an increased tumor incidence seen in addicted patients.

1. Introduction

Opium is a mixture of substances that contains 8–17% morphine, 1–10% noscapine, 0.5–1.5% papaverine and 0.7–5% codeine [1]. Morphine is the main component of opium and has several controversial effects on the immune systems including induction of apoptosis in immune cells, typical atrophy in thymus and spleen [2] as well as suppression of mitogen-stimulated proliferation of B and T lymphocytes [3]. It seems that, morphine can disturb the functions of T lymphocytes, macrophages, the cytotoxicity function of NK cells and has an inhibitory effect on cytokines secretion [4,5]. Interestingly, the risk of bacterial and viral diseases including HIV, hepatitis B and C and autoimmune diseases is prevalent among morphine addicts especially venous drug abusers [6,7]. Researchers believe that opioids can affect the immune system directly, because all three kinds of opioid receptors (κ, δ, μ) are expressed on the surface of lymphocytes and mononuclear phagocytes [8]. In addition, many opium derivatives influence the production and release of cytokines such as IL-4 and IFN-γ [9]. Controversially, morphine, noscapine, papaverine and codeine, the main components of opium, can be considered as inducers and inhibitors of inflammation [10]. Morphine can effect cytokine secretion such as IFN-γ by human PBMCs, T lymphocytes and monocytes [11]. Moreover, Chao et al., identified that morphine induces TGF-β secretion by human PBMCs [12]. Some *in vitro* studies also showed that heroin (diacetylmorphine) had an inhibitory effect on T lymphocytes proliferation and IFN-γ secretion after exposure to the concanavalin A stimulator [13]. Svetlecic et al., reported that papaverine administration lead to significant increase of IL-4, IL-13 and TGF-β levels [14].

All of the mentioned studies have evaluated the effects of opium derivatives such as morphine, heroin and papaverin on the immune system *in vitro* and in animal models. To the best of our knowledge, *in vivo* studies measuring the effects of opium on cytokine levels in humans has rarely been measured. Furthermore, opium is potentially a complex mixture of 20 alkaloids and 70 different components [15,16] and it will be difficult to ascertain

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which components are directly influencing the modulation of the immune system. To initiate the human studies, we decided to measure the effects of chronic opium consumption on the circulating levels of IL-6, IFN-γ, IL-4 and TGF-β in humans in comparison to non-opium-addicted individuals. In addition, the effects of opium on cytokine secretion of lymphocytes derived from peripheral blood of opium-addicted and non-opium-addicted individuals in *in vitro* conditions was also assessed.

2. Materials and methods

2.1. Subject selection

Subjects consisted of 30 male opium-addicted individuals for the case studies and 30 male non-opium-addicted individuals as controls. All subjects were aged from 19 to 56. Exclusion criteria included any kind of disease (either symptoms or diagnosis), consumption of any medication, consumption of other component of opium (Heroin, morphine, *etc.*), cigarette smoking and withdrawal drugs such as methadone or other withdrawal regimes. All case subjects had been using opium at levels more than 500 mg per day continuously for at least 1 year prior to sample preparation. The method that was used by all subjects was smoking opium using and opium pipe (Bafur). For each subject in the case group a person with similar attributes regarding age, residency and BMI with no addiction to opioid drugs was selected. This study was approved by the ethical committee of the Rafsanjan University of Medical Sciences and written informed consent was obtained from all of participants, both opium-addicted and control subjects, prior to sample collection.

2.2. Isolation and culture of lymphocytes

Peripheral blood samples were obtained from the subjects at 8–9 a.m., before consuming opium and plasma was separated and stored at −80 °C for future analysis. In order to isolate lymphocytes, fresh whole blood (20 mL) was collected in plastic tubes containing EDTA as an anticoagulant. The blood was diluted (1:2) with phosphate buffered saline (PBS; 0.1 M Sodium phosphate, 0.14 M NaCl, pH 7.4), layered onto a ficoll-paque and then separated by centrifugation at low speed (2000 rpm) at 25 °C for 30 min. The enriched lymphocyte layer (interphase layer) was collected into a falcon tube. The lymphocytes were washed twice with PBS. The lymphocytes were counted with the viable dye, 0.1% trypan blue (Sigma).

Opium was donated by the anti-drug section of the Kerman Police (Iran). Based on their information the origin of the opium was Helmand in Afghanistan. Analysis of this opium by GC–mass spectrometry revealed that more than 30% consisted of alkaloids, the most abundant of these were; 16% morphine, 5.5% codeine, 4.4% thebaine and 3.2% was papaverine. The rest consisted of non-alkaloidal organic and non-organic substances, 13.5% was determined to be water (moisture). From the stock of 2.86 g/mL opium in RPMI1640 solution, a dilution of 2.86×10^{-5} g/mL was prepared in RPMI1640 medium for the culture of lymphocytes. These calculations were based on the pharmacological dose and the effective concentrations of morphine on the Jurkat cell line [17] based on the assumption that opium contains 16% morphine. Lymphocytes (separated from the peripheral blood of samples) were used at a final concentration of 1×10^6 cells per well in 12 well culture plates in the RPMI 1640 media containing 10% FBS (Invitrogen, USA) and were exposed to concentrations of 2.86×10^{-5} g/mL opium or culture medium alone for periods of 48 h (three wells for each condition). At the end of incubation, the supernatant was used for cytokine assays.

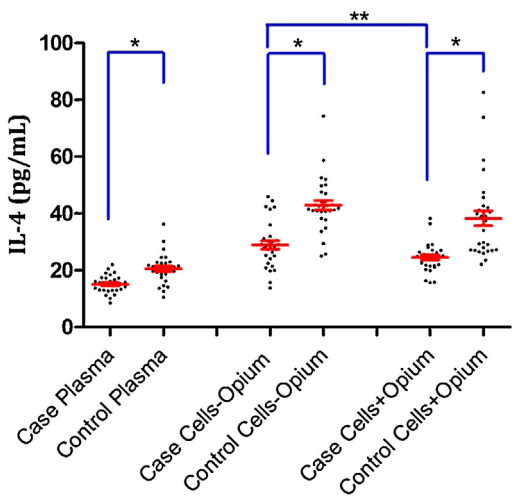

Fig. 1. Concentrations of IL-4 in plasma and the supernatant of lymphocytes cultured from cases and controls after 48 h incubation with and without opium (2.86×10^{-5} g/mL). Matched evaluations for cases *versus* controls comparing plasma levels, supernatants from lymphocytes cultured with opium (2.86×10^{-5} g/mL) and without opium. Statistical significance is shown above the scatter plots; * $P < 0.0001$ and ** $P = 0.0139$.

2.3. Cytokine assays

The levels of cytokines (IL-6, IFN-γ, IL-4 and TGF-β) derived from plasma and the supernatant of cultured lymphocytes was measured by ELISA (R&D Systems, USA) according to manufacturer's guidelines. The sensitivity of the kits was 2 pg/mL and inter- and intra-assay assessments of reliability of the kit were conducted.

2.4. Statistical analysis

Results are presented in scatter plots showing the mean ± standard errors (SE). All analyses were performed by SPSS (version 16; SPSS Inc.). One-way analysis of variance (ANOVA) followed by Tukey *post hoc* tests was done for intra group comparison of cytokines levels in plasma and supernatants of cells cultured with and without opium. Comparisons of mean values between two groups (*i.e.* opium-addicted and non-opium-addicted individuals) were performed by two tailed *t*-test using Prism GraphPad Version 5.03 (GraphPad Software Inc.) and a *P* value ≤0.05 was considered as statistically significant.

3. Results

3.1. IL-4 expression levels in vivo and in vitro

The results demonstrated that the levels of IL-4 in plasma from cases (15.11 ± 0.5561 pg/mL) was lower than controls (20.57 ± 0.9420 pg/mL) and these differences were significant, $P < 0.0001$ (Fig. 1). The levels of IL-4 in the supernatants of lymphocytes taken from cases (28.92 ± 1.485 pg/mL) was also lower than controls (42.95 ± 1.721 pg/mL) after 48 h incubation and these differences were also significant, $P < 0.0001$ (Fig. 1). IL-4 levels from lymphocytes from cases (24.51 ± 0.9073 pg/mL) were less than controls (38.33 ± 2.605 pg/mL) after incubation with 2.86×10^{-5} g/mL opium for 48 h and these differences were significantly different, $P < 0.0001$ (Fig. 1). We also sort to evaluate the effects of 48 h of opium treatment (2.86×10^{-5} g/mL) on IL-4 secretion into the medium of cultured lymphocytes originating from cases and controls (Fig. 1). Lymphocytes from cases were responsive to opium treatment decreasing from 28.92 ± 1.485 pg/mL to 24.51 ± 0.9073 pg/mL in treated cells, $P = 0.0139$. There were