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The Co-administration of Nicotine/ Marijuana and Morphine Changes Pro-inflammatory Cytokines in Rats

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Abstract

Background: Simultaneous co-consumption of abused substances is common among drug users. This study examined the effects of co-administration of marijuana/ nicotine and morphine on some cytokines in rats.

Method: Ninety eight rats were randomly divided into fourteen experimental groups including control (saline 1 ml/kg, i.p.), morphine (1, 3 and 5 mg/kg, i.p.), nicotine (0.5, 2 and 4 mg/kg, i.p.), marijuana (2.5, 5 and 10 mg/kg, i.p.) and the combination groups in which the rats received the combination of either effective or sub-effective doses of nicotine/ marijuana and morphine. Inflammation was induced via formalin injection into the left hind paw of all the control and the treated rats. The serum concentrations of some cytokines (TNF-α, IL-1, and IL-6) were measured by using an enzyme-linked immune-sorbent assay (ELISA) technique.

Results: A significant reduction in TNF-α, IL-1, and IL-6 concentration was observed in marijuana, nicotine and morphine treated rats. Also, the co-administration of effective doses of marijuana/ nicotine and morphine caused a significant reduction in cytokines, indicating either an additive or a synergistic effect.

Conclusion: The clinical application for the combined use of these substances has not been determined yet and further research is needed to clarify the efficacy, safety and tolerability of these combinations in inflammatory process.

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Introduction

Simultaneous polysubstance abuse such as use of marijuana, alcohol, opioids, nicotine and psychostimulants (ecstasy, methamphetamine and methylphenidate) is a common phenomenon among adolescents (1). The combined use of abused substances results in pharmacological effects beyond the effects of each substance alone; for example, nicotine can increase the intake of other abused substances (2). Also, previous reports have indicated that most alcoholics are also nicotine dependent and vice versa (3).

It is well known that cigarette smoking is associated with the increased risk of a number of diseases including lung cancer

and respiratory infections caused by the production of reactive oxygen species (ROS). Paradoxically, recent studies have suggested the anti-inflammatory effects of nicotine, the main constituent of cigarette (4). Nicotine as an agonist of acetylcholine receptor (α7-nAChR) subunit decreases pro-inflammatory mediators (4, 5). Direct activation of nicotinic acetylcholine receptors on immune cells can modulate the release of inflammatory cytokines in some inflammatory diseases. So, due to their anti-inflammatory effects, selective agonists for nicotinic receptors may be suggested as a pharmacological therapy in some inflammatory diseases such as ulcerative colitis and obesity (4-6). Kalayciyan *et al*. (2007) have reported that nicotine significantly decreases the release of inflammatory cytokines including interleukins (IL- 8 and IL-6) by human keratinocytes and dermal microvascular endothelial cells (HMEC-1) in patients with Behçet's disease (7). Nizri *et al.* (2009) have reported the activation of the cholinergic anti-inflammatory system by nicotine reduced T cell proliferation, as well as the production of tumor necrosis factor-α (TNF-α) and Th17 cytokines (IL-17, IL-17F, IL-21, and IL-22) which resulted in suppression of neuro-inflammation (8).

Contrary to previous reports, Ebrahimpour *et al.* (2019) have reported that nicotine stimulates the growth factors including fibroblast, platelet-derived, and vascular endothelial growth factors and downregulates anti-inflammatory microRNAs in lung cells which accelerate the disease process in idiopathic pulmonary fibrosis (9).

Marijuana refers to dried leaves, flowers, stems, and seeds of the *Cannabis sativa* and contains the psychotropic tetrahydrocannabinol (THC) and non- psychoactive substances such as cannabidiol (CBD), and cannabinol (CBN). In a preliminary study on limited sample in Iran, the prevalence of cannabinoid use was 0.6% (10). There is a growing increase in the prevalence of marijuana use in the United States among youths (11) and in Iran, too, after cigarette and alcohol, marijuana is the drug commonly used among adolescents (12). Short- term memory impairment, anxiety, paranoid thoughts, impairment in driving activities and increased heart rate are among the main adverse effects of marijuana or THC consumption (13, 14). However, the beneficial features of THC include its anti-inflammatory effects resulted from the reduction of cytokine release that causes enhancing of wound healing process, and anti-proliferative, anti-metastatic and apoptotic effects (15, 16). In SU *et al.* study (2012), activation of cannabinoid CB2 receptors significantly reduced the levels of IL-1β, IL-6 and tumor necrosis factor-α (TNF-α) in inflamed skin tissues (17). Nagarkatti *et al.* (2009) have reported the potential use of cannabinoids as a new class of anti-inflammatory agents (18). The anti-inflammatory property of marijuana (Cannabis sativa) is mainly mediated through its Cannabidiol (CBD) content as the non-psychoactive component of marijuana (19).

Opioid drugs are mainly used as potent analgesics for the treatment of constant and severe pain; however, opioid drugs are not currently used in the treatment of inflammatory diseases because their anti-inflammatory effects have not been completely recognized yet. The pharmacological effects of opioids are mediated by three major types of opioid receptors including μ (mu), k (kappa) and δ (delta) opioid receptors. The μ receptors are mainly involved in supraspinal and spinal analgesia, k receptors may be involved in opioid anti-inflammatory responses and δ receptors modulate hormone and neurotransmitter release (20, 21). Previous studies have showed that morphine, the prototypic opioid agonist, causes the anti-

inflammatory effects by activation of peripherally selective k-opioid agonists (22). Walker *et al.* (2003) demonstrated that k-opioids may act directly on inhibit cytokine release from immune cells (23). However, opioid use is consistently associated with an increased morbidity and mortality rate due to infections in both animals and heroin addicts (23, 24). In addition to their several well-known adverse effects, the opioid immunosuppressive effects may have important clinical implications for the individuals who use opioids for long-term, either for pain treatment in cancer or as addicts. Previous animal and human studies demonstrated the inhibitory effects of opioids, including morphine effect on immune responses (23). The opioid inhibitory effects on humoral and cellular immune responses are probably mediated through a decrease in antibody production, suppression of natural killer cell activity and suppression of cytokine production (24, 25). Also, contrary to previous results, morphine increases neuro-inflammation in human astrocytes and microglia of autophagy deficient mouse (26).

Although the anti-inflammatory effects of marijuana (Cannabis sativa) and especially Cannabidiol (CBD), as its main non- psychoactive component has been well documented, the inflammatory and anti-inflammatory properties of morphine and nicotine are controversial. Since the combined use of these substances is common among substance abusers and because, to the best of our knowledge, there was no reported document about the effects of combined administration of either nicotine or marijuana and morphine on inflammatory parameters, this study was conducted to evaluate the effects of nicotine, marijuana, morphine and their combination on some cytokines in rats.

Materials and Methods

Animals

Ninety-eight adult male Wistar rats, weighing 200-250g, with the age of 8-10 weeks, were purchased from the animal house of Kerman School of Medicine (Kerman, Iran) and housed 4 per cage. The animal house condition was maintained in the constant temperature of 23±2 ºC, humidity of 50-62% and 12 h light/dark cycle (lights on at 8.00AM). Animals had free access to food and tap water.

Ethical Guidelines

The protocol was conducted based on the approved ethical guidelines (NIH, publication no. 85-23, revised 1985; European Communities Directive 86/609/EEC) and approved by the Committee on the Ethics of Animal Experiments at Kerman University of Medical Sciences (Ethic code: IR.KMU.REC.1397.211).

Drugs

Nicotine (Sigma, England) and morphine (Temad Pharmaceutical Co, Iran) were used in this study. Marijuana was provided by the Police Headquarters for Combating Narcotics in Kerman Province, Iran. All compounds were dissolved in normal saline and all injections were done via intraperitoneal route.

Diagnostic ELISA kits were purchased from ZellBio GmbH, Ulm, Germany. The applied kits were: Tumor Necrosis Factor alpha (TNF-α), (cat.no. ZB-10764C-R9648, sensitivity: 46.88 pg/mL and detection range: 78.13~5000 pg/mL), Interleukin6 (IL-6), (cat.no. ZB-10135C -R9648, sensitivity: 7.50 pg/mL and detection range: 12.50~800 pg/mL), and Interleukin 1(IL-1) (cat.no. ZB-10107C -R9648,

sensitivity = 46.88 pg/mL and Detection Range: 78.13~5000 pg/mL).

Inflammation induction

Formalin injection into the paw of laboratory animal is a well-known method for induction of inflammation (27). Animals were acclimated to the laboratory conditions for 30 min. Then, 50 µl of 2.5% formaldehyde (Merck, Germany) solution was injected into the sub-plantar region of the left hind paw using a 30-gauge needle (U-100 insulin syringe, Yazd, Iran). The control group received normal saline. Then, the rats were placed immediately in the plexiglass chamber (25×25×40cm) with a 45º mirror located under it. The rats were observed for 60 minutes. Inflammation was confirmed according to the animal nociceptive behaviors as flinching, shaking, licking and biting of the injured paw and combinations of them. Pain behaviors were categorized from no pain (score:0) to maximum pain (score:3) by using Dubuisson and Dennis (1977) behavioral pain scores (27). Every 15 min. maximum score was annotated. In the all animals, the maximum score as an index of inflammatory pain was achieved during one hour of observation.

Experimental groups

Inflammation was occurred in all animals and confirmed by the achieved maximum score. Rats received drugs or saline one hour after formalin induced inflammation and then they were randomly divided into the 14 experimental groups. Based on similar experimental studies, each group consisted of seven rats.

-Control group (group 1): rats received normal saline (1ml, i.p.).

-Morphine groups (group 2 to 4): rats received morphine (1, 3 and 5mg/kg, i.p.).

- Nicotine groups (group 5 to 7): rats received nicotine (0.5, 2 and 4 mg/kg, i.p.).

- Marijuana groups (group 8 to 10): rats received marijuana (2.5, 5 and 10 mg/kg, i.p.)

- Combination groups (group 11 to 14); rats received the combination of effective doses of nicotine + morphine (group 11) and marijuana + morphine (group 12) or sub-effective doses of nicotine + morphine (group 13) and marijuana + morphine (group 14).

Procedure

All the experiments were done between 8 AM to 1 PM and in the room temperature of 23±2 ºC. The selections of doses were based on similar previous studies (5, 18, 28) and multiple scale doses of morphine (1,3,5 mg/kg), nicotine (0.5,2,4 mg/kg) and marijuana (2.5,5,10 mg/kg) were used to determine effective and sub-effective doses.

Initially, the first ten groups were randomly selected and entered in the study to determine the effective dose of morphine, marijuana and nicotine for future four combinational groups (11 to 14). The selections of doses were based on literature review and multiple scale doses to determine effective and sub-effective doses (By definition, the effective dose is the dose that makes significant change compared to the control and sub-effective is the dose immediately before the effective dose.) One hour after formalin injection, the animals received drugs intraperitoneally and one hour after the drug injection, the rats received ketamine and xylazine and under mild anesthesia, they were decapitated and whole blood (trunk blood) was collected in a tube. Then, without delay, the blood of each rat was

centrifuged (Heraeus, Germany) at 4000 rpm for 15 min and the serum was collected in micro tube (coagulant tubes) and was frozen in the temperature of -70 ºC for the analysis of TNF-α, IL-1, and IL-6.

The serum concentration of cytokines (TNF-α, IL-1, and IL-6) was measured by using an enzyme-linked immune sorbent assay (ELISA) technique. The samples were codified so the researcher who computed Elisa was not aware of the type of drug in samples. Also, data analyzer was blinded to the treatment group.

Statistical analysis

Data were analyzed using SPSS software version 21 (Chicago, Illinois, USA). Kolmogorov-Smirnov Test was used to test the group normality. Data were analyzed by one-way analysis of variance (ANOVA) followed by Post hoc Tukey's test for multiple comparisons. Data were expressed as the mean ± SEM of 7 rats in each group. A P-value smaller than 0.05 was considered significant.

Results

The effects of morphine and nicotine and marijuana on inflammatory cytokines

As it is seen in Fig 1A-B-C, morphine in dose of 5mg/kg significantly reduced all three parameters of IL-1 ($p<0.05$), IL-6 ($p<0.005$) and TNF-α ($p<0.05$) compared to controls while dose of 3mg/kg caused a significant decrease of only IL-6 ($p<0.05$) concentration compared to the control group and dose of 1mg/kg reduced none of the three parameters.

Morphine reduced IL-6 concentration in a dose dependent manner since morphine 1mg/kg had no effect, but morphine 3 mg/kg and 5mg/kg decreased IL-6 significantly ($p<0.05$ and $p<0.005$ respectively) compared to the control group (Fig 1B).

Fig 1A-B-C showed that the high dose of nicotine (4mg/kg) significantly reduced the concentration of IL-1 ($p<0.05$), IL-6 ($p<0.005$) and TNF-α inflammatory cytokine ($p<0.05$).

Marijuana in the doses of 2.5 and 5mg/kg had no effect on IL-1 and TNF-α while in dose of 10 mg/kg significantly ($p<0.05$) decreased the concentration of IL-1 and TNF-α compared to the control group (Fig 1A and Fig 1C). Marijuana (5 and 10mg/kg) significantly ($p<0.05$) reduced the concentration of IL-6 compared to the control group (Fig 1B).

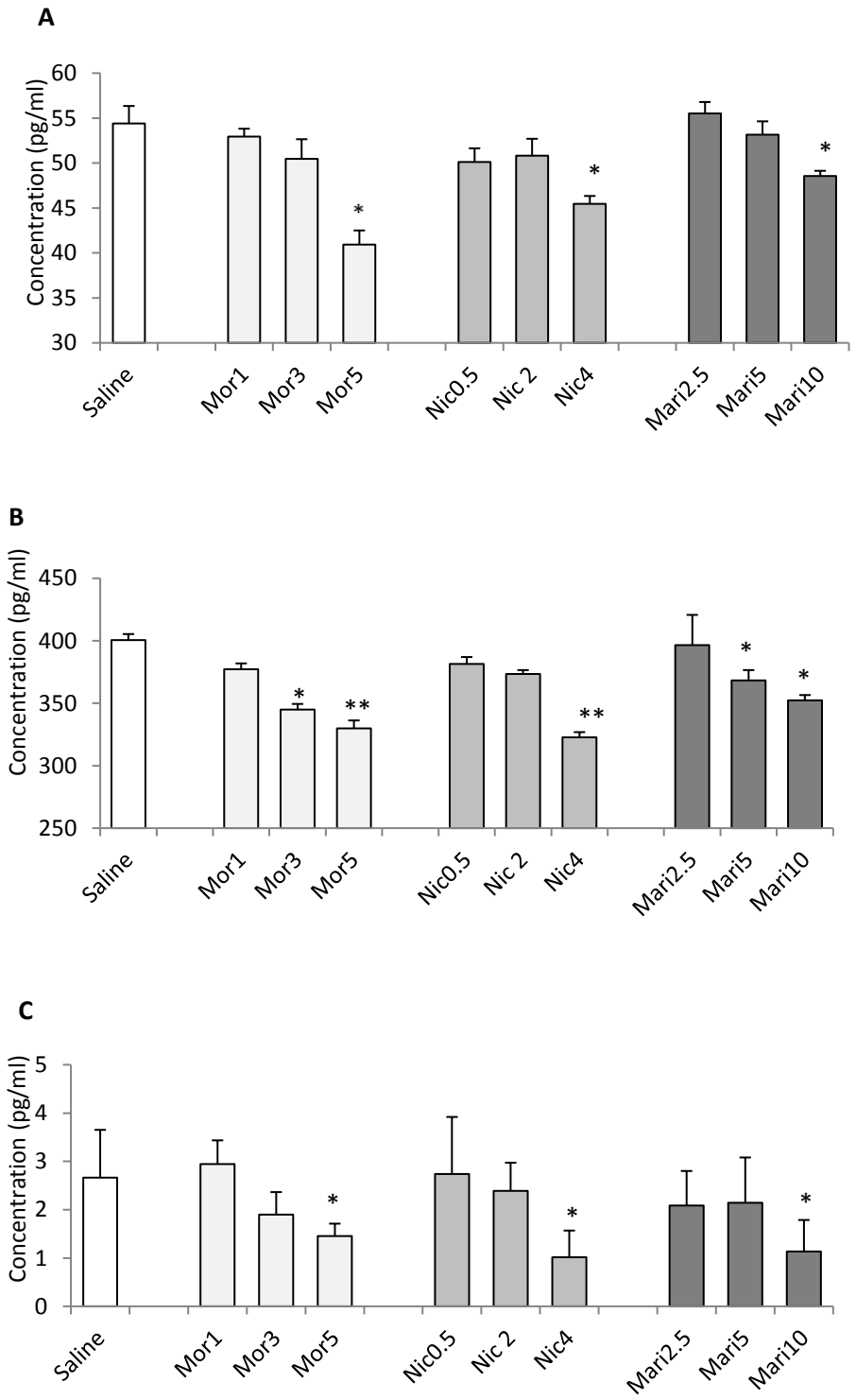

Figure 1. The effects of morphine (1, 3 and 5 mg/kg, i.p.), nicotine (0.5, 2 and 4 mg/kg, i.p.), and marijuana (2.5, 5 and 10 mg/kg, i.p.) on cytokine levels of rats. (A): IL-1 concentration, (B): IL-6 concentration and (C): TNF-α concentration. Data represent means ± SEM of 7 rats.

* $p<0.05$ compared to the control group, ** $p<0.005$ compared to the control group, Mor: morphine, Nic: nicotine, Mari: marijuana

The effects of co-administration of effective doses of nicotine and marijuana with morphine on inflammatory cytokines

The results showed that the co-administration of effective dose of nicotine (4mg/kg) and morphine (5mg/kg) caused a significant decrease in IL-1 concentration compared to control ($P<0.005$), nicotine ($p<0.05$) and morphine alone ($p<0.05$) (Fig 2A).

Also, the combination therapy of nicotine (4mg/kg) and morphine (5mg/kg) significantly decreased IL-6 concentration, compared to control ($P<0.005$), nicotine ($p<0.05$) and morphine alone ($p<0.05$) (Fig 2B). The combined administration of effective dose of morphine with nicotine caused significant changes in TNF-α concentration, compared to control ($P<0.005$), morphine ($p<0. 05$) and nicotine groups ($p<0. 05$) (Fig 2C). The co-administration of effective dose of marijuana (10mg/kg) with morphine (5mg/kg) revealed a significant decrease in IL-1 concentration compared to control ($P<0.005$), morphine ($p<0.05$) and marijuana ($p<0.05$) alone (Fig 2 A). Also, the co-administration of effective doses of marijuana (10mg/kg) and morphine (5mg/kg) showed a significant decrease in IL-6 concentration compared to control ($P<0.005$), morphine ($p<0.05$) and marijuana ($p<0.05$) alone (Fig 2 B). The combined administration of effective dose of marijuana (10mg/kg) with morphine (5mg/kg) showed a significant decrease in TNF-α concentration compared to control ($P<0.005$), morphine ($p<0.05$) and marijuana ($p<0.05$) alone (Fig 2C).

The effects of co-administration of sub-effective doses of nicotine or marijuana with morphine on inflammatory cytokines

Results indicated that co-administration of sub-effective dose of nicotine (2mg/kg) with morphine (1 mg/kg) had no significant effects on IL-1, IL-6 and TNF-α concentrations (Fig 3). As well, the co-administration of sub-effective dose of marijuana (2.5 mg/kg) with morphine (1mg/kg) revealed no significant effects on IL-1, IL-6 and TNF-α concentration (Fig 3).

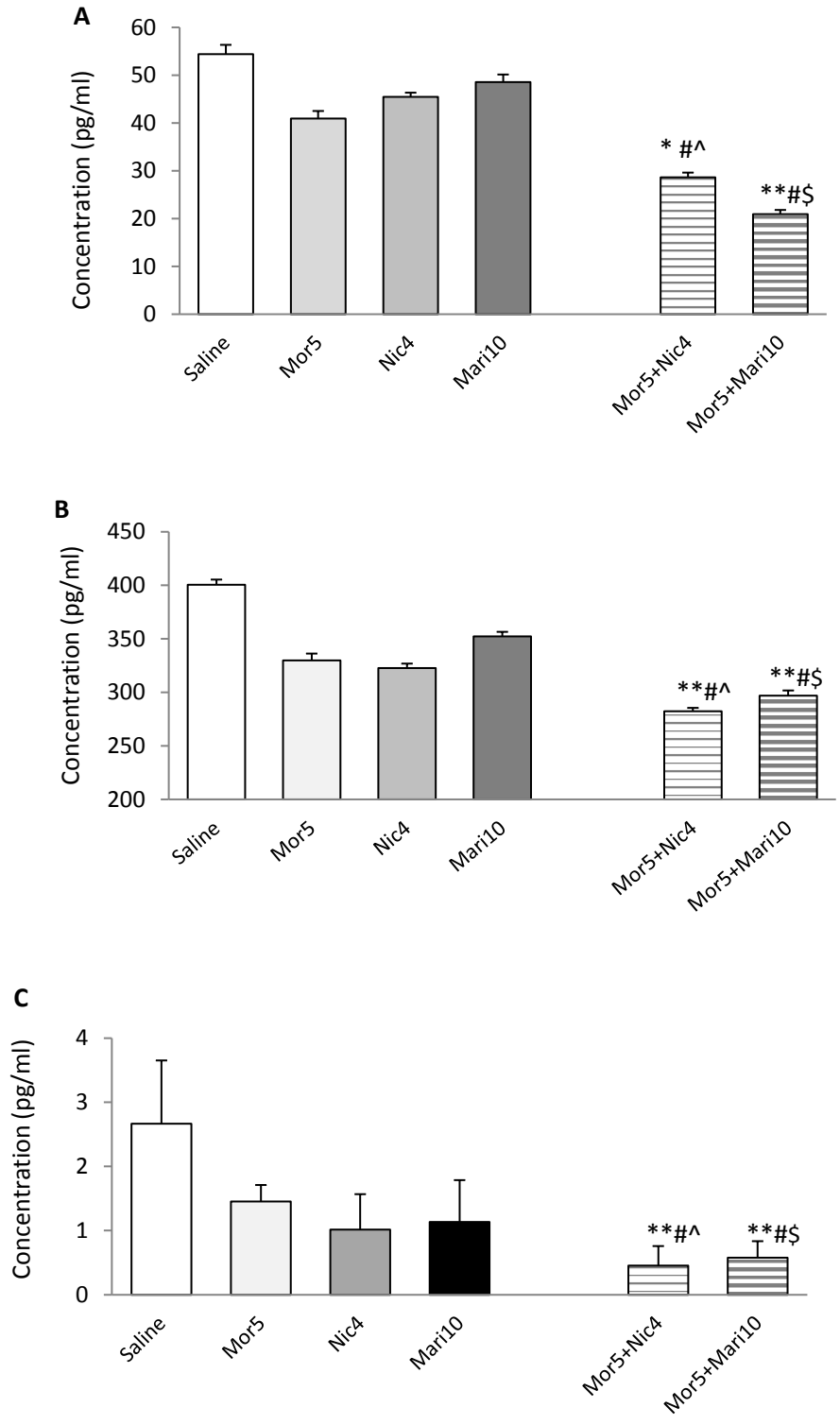

Figure 2. The effects of co-administration of effective doses of nicotine Nic (4mg/kg, i.p.) or marijuana Mari(10 mg/kg, i.p.) with morphine Mor (5mg/kg, i.p.) on (A): IL-1, (B): IL-6 and (C): TNF-α concentration of rats. Data represent means ± SEM of 7 rats.

* $p<0.05$ compared to control, ** $p<0.005$ compared to control, ^ $p<0.05$ compared to morphine alone, # $p<0.05$ compared to nicotine alone, $ $p<0.05$ compared to marijuana alone, Mor: morphine, Nic: nicotine, Mari: marijuana

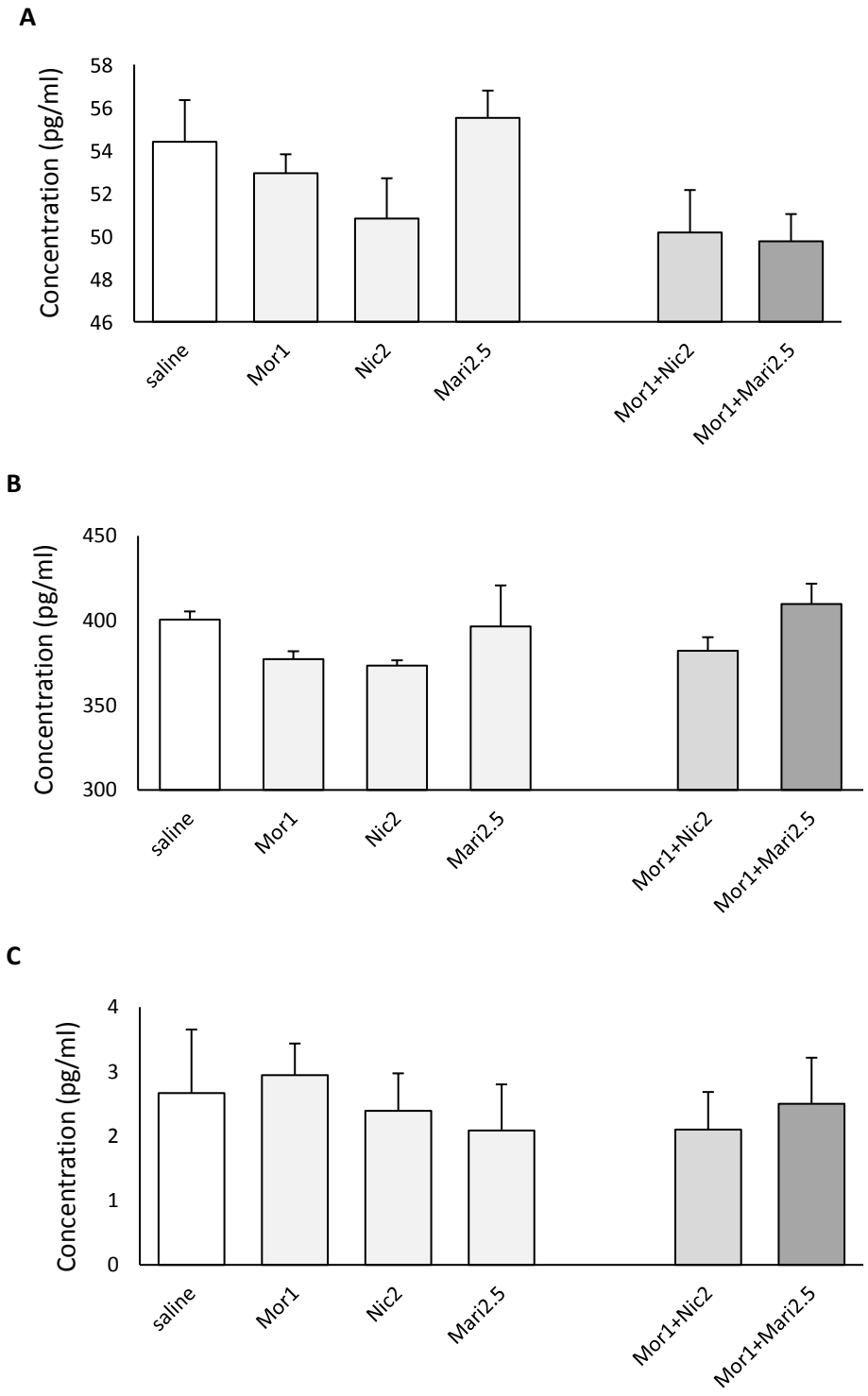

Figure 3. The effects of co-administration of ineffective doses of nicotine Nic (2mg/kg, i.p.) and morphine (1mg/kg, i.p.) and marijuana (2.5 mg/kg, i.p.) on (A): IL-1, (B): IL-6 and (C): TNF-α concentration of rats. Data represent means ± SEM of 7 rats.

Mor: morphine, Nic: nicotine, Mari: marijuana

Discussion

This study examined the effects of nicotine/ marijuana with morphine on some cytokines in rats**.** In agreement with previous studies, present findings demonstrated that nicotine, marijuana and morphine can reduce the cytokines IL-1, IL-6, and TNF-α (7, 25, 29). In general, cytokines can be divided into those with predominantly pro-inflammatory actions TNF-α, interleukin IL-1, IL-6, and IL-8 and those with anti-inflammatory actions IL-1 receptor antagonist, IL-10, IL-13 (30, 31). According to the results, marijuana administration was associated with a reduction in cytokines IL-1, IL-6, and TNF-α in a dose dependent manner which is comparable to some previous reports (19).

Marijuana and its main components (mainly THC & CBD) possess anti-inflammatory properties (32); however, THC demonstrated both pro-inflammatory and anti-inflammatory effects (18). Previous studies indicated that THC administration has been associated with significant reduction in cytokines (IL-1α, IL-1β, IL-6 and TNF-α) in lipopolysaccharide (LPS)-stimulated cultured microglia cells of rat (18).

Izzo&Camilleri (2009) reported that cannabinoids exert beneficial effects in intestinal inflammation and cancer via direct or indirect activation of CB1 and/or CB2 receptors (16).

The beneficial anti-inflammatory effects of cannabinoids are mediated via both central and peripheral CB receptors (33, 34). Ribeiro (2012) reported that cannabidiol (CBD), a non-psychotropic component of marijuana (Cannabis sativa), has potent immunosuppressive and anti-inflammatory properties through significant reduction of pro-inflammatory cytokines (TNF-α and IL-6) in a murine model of LPS-induced acute lung injury (35). Cooper *et al*., reported that co-consumption of smoked cannabis with oxycodone enhanced anti-nociceptive effects of the sub-effective dose (sub-threshold) of oxycodone, indicating a synergistic effect (29).

In agreement with previous reports indicating nicotine anti-inflammatory activities, Yoshikawa *et al.* (2006) showed that pretreatment with low-dose nicotine caused inhibition of pro-inflammatory mediators (TNF-α) and PGE2 (5). De Simone *et al.* showed that nicotine reduced the LPS-induced release of TNF-α from the rat microglia cells in a dose dependent manner (36). In another study, sub-chronic injections of nicotine reversed the morphine induced amnesia in mice (37).

In relation to the morphine anti-inflammatory properties, Philippe *et al.* (2003) showed that mu opioid receptor is involved in control of gut inflammation via the reduction of cytokine production (TNF-α, IL-4) and T cell proliferation in an experimental model of colitis in mice (38). Stein and Küchler reported that peripherally active opioids are useful for the treatment of inflammatory pain in arthritis and promote wound healing in burns, skin grafts, and chronic wounds (39). Spinal pro-inflammatory cytokines could act as a powerful pain-enhancing signal which causes a significant reduction of opioid analgesia in both acute and chronic inflammatory pain (40). Finley *et al.* reported that opioids can modulate inflammation and immune system via the regulation of cytokine, chemokine, and cytokine receptor expression (41). Contrary to our results, other investigators reported a significant increase in cytokines after chronic morphine treatment in rats (42). Several other studies have reported that morphine exacerbate pain by its pro-inflammatory and pro-nociceptive effects. The precise underlying mechanism(s) is not known yet, but it is proposed that morphine potentiate the injury-induced inflammatory signaling in the central nervous system (43, 44).

Our results showed that the co-administration of effective doses of nicotine and marijuana with morphine significantly decreased cytokines IL-1, IL-6 and TNF-α concentrations, compared to nicotine, marijuana and morphine alone, which could be the novel finding of this study and whether this interaction indicates an additive or synergistic effect was not determined. Although nicotine, marijuana and morphine possess anti-inflammatory properties, to our knowledge, there is no documented data on the effects of combined use of nicotine or marijuana with morphine on inflammatory responses and most of the previous studies have focused on the analgesic effects of combined use of these compounds. In consistency with our results, previous studies have confirmed the synergistic interactions between cannabinoid and opioid induced analgesia (45, 46). In Nielsen *et al.* (2017) study, co-administration of cannabinoids with opioids was associated with a significant reduction for opioid requirements in the treatment of acute pain, and the interaction was a synergistic effect (٤٦). Boehnke *et al.* (2016) reported that medical use of cannabis was associated with a significant reduction of opioid use in patients with chronic pain (4٥). In another study, inhaled vaporized cannabis in patients with chronic pain was associated with augmentation of the analgesic effects of opioids indicated a synergistic analgesic effects without any significant change in plasma opioid levels (4٧). In this study, nicotine administration was associated with a dose dependent reduction in inflammatory cytokines which is comparable to previous reports (٤). However, recent researches have showed that nicotine accelerates the inflammatory responses in idiopathic pulmonary fibrosis (٩).

Also, previous studies have indicated that nicotine potentiates the analgesic effects of opioids via the nicotinic acetylcholine receptor (nAChR) -induced release of endogenous opioids (3,4٨,٤٩). The results of another study indicated that tobacco smoking during pregnancy in opioid-dependent pregnant women was associated with a greater risk for additional adverse events in newborn, indicating the additive or synergistic effects of the combined use of tobacco and opioids (5٠). In a previous study, prolonged co-consumption of morphine and nicotine decreased Plasma rennin activity (PRA) and blood pressure and increased the serum concentration of nitric oxide (NO) in hypertensive rats (5١). Abreu-Villaca *et al.* (2013) reported that combined exposure to nicotine and ethanol was associated with lower anxiety levels in elevated plus maze in female Swiss mice (5٢).

In summary, in the present study, nicotine, marijuana and morphine decreased inflammatory cytokines IL-1, IL-6 and TNF-α concentration. Also, the combined use of either nicotine or marijuana with morphine was associated with a significant decrease in cytokines concentration, indicating either additive or synergistic effects. These findings may have important clinical implications in the treatment of patients suffering from inflammatory conditions. Further research is needed to clarify the efficacy, safety, tolerability and mechanism of active principles of the combined medications using marijuana or nicotine with opioids in inflammatory process.

Limitations

Cytokines can exert both pro-inflammatory and anti-inflammatory actions. This study evaluated the effects of nicotine, marijuana and their co-administration with morphine on pro-inflammatory cytokines, however, their effects on anti-inflammatory cytokines was not determined. Also, this study showed the effects of acute administration of nicotine,

marijuana alone and their combination with morphine on some pro-inflammatory cytokines. Since these substances are used for chronic periods by substance abusers, it is of a great importance to evaluate the effects of chronic administration of nicotine marijuana, morphine and their co-administration on both pro-inflammatory and anti-inflammatory cytokines in both animals and substance abusers.

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