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Acute Immunomodulatory Effects of Fentanyl and its Three New Analogues in Swiss Albino Mice

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ABSTRACT

Fentanyl is a potent synthetic opioid analgesic. However, due to its several limitations, new analogues are being synthesised for better pain management. We have earlier reported the synthesis and bio-efficacy of fentanyl and its eight new analogues (**1-8**) in mice. Among eight analogues tested, *N*-(1-(2-phenoxyethyl)-4-piperidinyl) propionanilide (**2**), *N*-isopropyl-3-(4-(N-phenylpropionamido)piperidin-1-yl)propanamide (**5**), and *N*-t-butyl-3-(4-(N-phenylpropionamido)piperidin-1-yl)propanamide (**6**) were found to be more effective and less toxic compared to fentanyl. Therapeutic efficacy of fentanyl and its analogues are known to be compromised due to many adverse effects, including alterations in the immune system. Therefore, the present study was undertaken to assess the acute effect of fentanyl and its three analogues (**2**, **5**, and **6**) on plasma levels of different pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and anti-inflammatory cytokines such as interleukin-10 (IL-10) at different time points. Mice were intraperitoneally treated with 0.50 LD_{50} of the compounds and cytokines were measured 1 h, 2 h, 4 h, and 24 h post-exposure. Compared to control, none of the treatments produced any change in TNF-α and IL-1β levels. However, IL-6 levels were significantly elevated between 1 h to 2 h post-exposure in fentanyl and analogue **2** treated groups. Further, IL-10 levels were found to be significantly increased in fentanyl, analogue **2**, and **6** treated groups at 1 h and 2 h post-exposure. Pre-treatment of naltrexone (opioid receptor antagonist) blocked the effects of fentanyl, confirming that its effects were opioid receptor- dependent. However, effect of naltrexone on analogue **2** and **6** was not conclusively evidenced, indicating that immunomodulatory changes caused by the analogues could have some additional implications as well. The present study reveals undesirable effects of fentanyl and its new analogues on cytokines homeostasis, thereby limiting their use in pain management.

Keywords: Fentanyl analogues; Pain management; Acute effect; Cytokines

1. INTRODUCTION

Management of excruciating pain in an out of hospital scenario is very crucial for the recovery of soldiers and their performance. Opioid analgesics are widely used for the treatment of severe and chronic pain. Fentanyl is a synthetic narcotic opioid analgesic agent, very similar to morphine. However, fentanyl has many advantages due to its rapid onset of action, short duration of action, minimal provocation of histamine release, and many-fold higher potency than morphine [1-2]. This drug is a strong agonist of μ-opioid receptor (MOR), which is widely implicated in surgical analgesia and sedation[3,4]. The clinical use of fentanyl remained restricted due to its undesirable effects on central nervous system (CNS), like respiratory depression, sedation, nausea, muscle rigidity, and tolerance and addiction after prolonged use[5,6]. In addition, they modulate immune functions and cause immunosuppression[6,7]. Morphine and related opioids have many diverse effects on the immune system including suppression of cell mediated, humoral-mediated, and natural (nonspecific) immunity. Many of the effects of opioids on immune system are indirect and appear to be mediated through the CNS, suggesting that alterations in opioid neurotransmission can alter the efficacy of the immune response[7-9]. Cytokines are low molecular weight proteins, which modulate cell growth, maturation, and intercellular communication of immune-competent cells[7]. Cytokines include chemokines, interferons, interleukins, lymphokines, and tumor necrosis factor. It has been reported that pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and anti-inflammatory cytokines such as interleukin-10 (IL-10) increase in patients with sepsis, trauma, and burns. Moreover, these cytokines are associated with the development of septic shock and organ dysfunction[10].

Synthesis and comparative bioassay of fentanyl and its 1-substituted analogues (**1-8**) in mice were earlier reported by us[11,12]. Out of these eight compounds, *N*-(1-(2-phenoxyethyl)-4-piperidinyl)propionanilide (**2**), *N*-isopropyl-3-(4-(N-phenylpropionamido)piperidin-1-yl)propanamide (**5**), and *N*-t-butyl-3-(4-(N-phenylpropionamido)piperidin-1-yl) propanamide (**6**) were found to be more effective and less toxic

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compared to fentanyl. Different opioids have different degree of immunomodulation. Therefore, evaluating each opioid's profile is important for appropriate analgesic selection[13]. The aim of the present study was to determine the acute effect of fentanyl and its three analogues viz., analogue **2, 5,** and **6** on plasma levels of different pro-inflammatory and anti-inflammatory cytokines at different time points, with a view to see the immunomodulatory effects of these compounds.

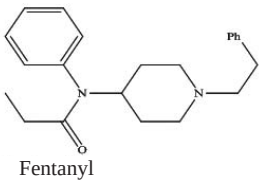
2. MATERIALS AND METHODS

2.1 Animals

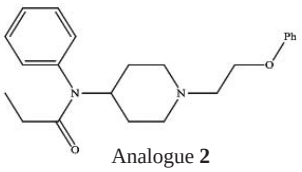
Male Swiss albino mice (25-30 g) were procured from the Animal Facility of Defence Research and Development Establishment (DRDE), Gwalior. The animals were housed in polypropylene cages on dust free rice husk as the bedding materials, with free access to food (Ashirwad Brand, Chandigarh, India*)* and water ad libitum. Prior to experiment, animals were randomised and acclimatised for seven days in controlled environmental conditions (22±2°C; relative humidity 40 % - 60 %) at a 12 h light/12 h dark cycle. The care and maintenance of the animals were as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Animal experiments were carried out with the approval of Institutional Animal Ethical Committee.

2.2 Chemicals

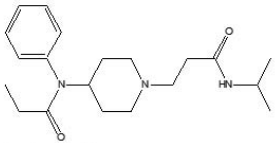
Naltrexone hydrochloride and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). Fentanyl and its 1-substituted analogues (**2, 5**, and **6**) were synthesised and characterised by IR, ^{1}H NMR, ^{13}C NMR, GC-MS and elemental analysis in the Synthetic Chemistry Division of DRDE, Gwalior as per the methods discussed elsewhere[11,12]. The structures of the compounds are as follows:



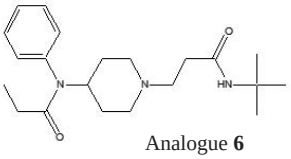
Fentanyl



Analogue **2**



Analogue **5**



Analogue **6**

2.3 Treatment

One hundred twenty mice were divided into five groups of twenty four animals each as follows: (1) Control (DMSO), (2) fentanyl (3) analogue **2** (4) analogue **5**, and (5) analogue **6**. All the compounds were dissolved in DMSO and administered intraperitoneally (i.p.) in a volume <10 ml/kg body weight. The doses of fentanyl and its analogues were equivalent to their respective 0.50 LD_{50} (i.p.) values, which were 8.75 mg/kg (fentanyl), 56.10 mg/kg (analogue **2**), 56.90 mg/kg (analogue **5**), and 53.65 mg/kg (analogue **6**)[11,12]. Six animals were randomly selected from each group for each time point and blood was collected at 1 h, 2 h, 4 h, and 24 h post-exposure for measurement of various cytokines. Blood was drawn from the retro-orbital plexus ether anesthetised animals using haparinised capillary. In a separate study, forty eight mice were divided into four groups of twelve animals each as follows: (1) Control (DMSO), (2) fentanyl (0.50 LD_{50}) + naltrexone, (3) analogue **2** (0.50 LD_{50}) + naltrexone, (4) analogue **6** (0.50 LD_{50}) + naltrexone. Naltrexone (10 mg/kg) was subcutaneously (s.c.) administered 30 min prior to treatment with fentanyl or analogues. Six animals were randomly selected from each group for each time point and blood was collected 1 h and 2 h post-exposure for measurement of cytokines. The dose, route, and time of naltexone hydrochloride were selected on the basis of previous study[9]. Furthermore, Houghtling and Bayer[9] reported that naltrexone pre-treatment had no effect on plasma cytokines. Therefore, in the present study a separate group for naltrexone was not used.

2.4 Measurement of Cytokines

Various cytokines such as tumor necrosis factor alpha (TNF-α; pg/ml), interleukin-1β (IL-1β; pg/ml), interleukin-6 (IL-6; pg/ml), and interleukin-10 (IL-10; pg/ml) were measured in blood plasma using Enzyme Linked Immunosorbent Assay (ELISA) kits (Thermo Scientific, Rockford, USA) as per manufacturer's protocol, using Multimode Microplate Reader (Synergy 4, BioTek Instruments, VT, USA).

2.5 Statistics

The results are expressed as mean ± SEM (n=6). The data were analysed by one way ANOVA followed by Dunnet's test. Statistical significance was drawn at *$p<0.05$ and **$p<0.01$ levels using SigmaStat software (Jandel Scientific Inc., CA, USA).

3. RESULTS

In the present study, the immunomodulatory effect of fentanyl and its analogues (**2, 5**, and **6**) was assessed by measuring both pro-inflammatory (TNF-α, IL-1β, IL-6) and anti-inflammatory cytokine (IL-10) in plasma of treated animals at various time points. Figures 1 and 2 show the effect of fentanyl and its analogues on TNF-α and IL-1β levels, respectively. There was no significant difference observed in TNF-α and IL-1β levels between the control and treated groups. The IL-6 levels were significantly increased in fentanyl and analogue **2** treated groups 1 and 2 h post-exposure as shown in Fig. 3. The IL-10 levels were found to be significantly increased in fentanyl, analogue **2** and **6** treated groups 1 and 2 h post-exposure as shown in Fig. 4.

In order to determine whether the elevation of plasma IL-6 (fentanyl, analogue **2**) and IL-10 (fentanyl, analogue **2** and **6**) was mediated through the activation of opioid receptors, the animals were pre-treated (-30 min) with opioid receptor antagonist naltrexone[9]. Pre-treatment of naltrexone was found to block the elevation of IL-6 as shown in Fig. 5 and IL-10 as shown in Fig. 6. in fentanyl treated group. However, the increased levels of IL-6 in analogue **2** treated as shown in Fig. 5 and IL-10 in analogue **2** and **6** treated groups as shown in Fig. 6 continued to persist.

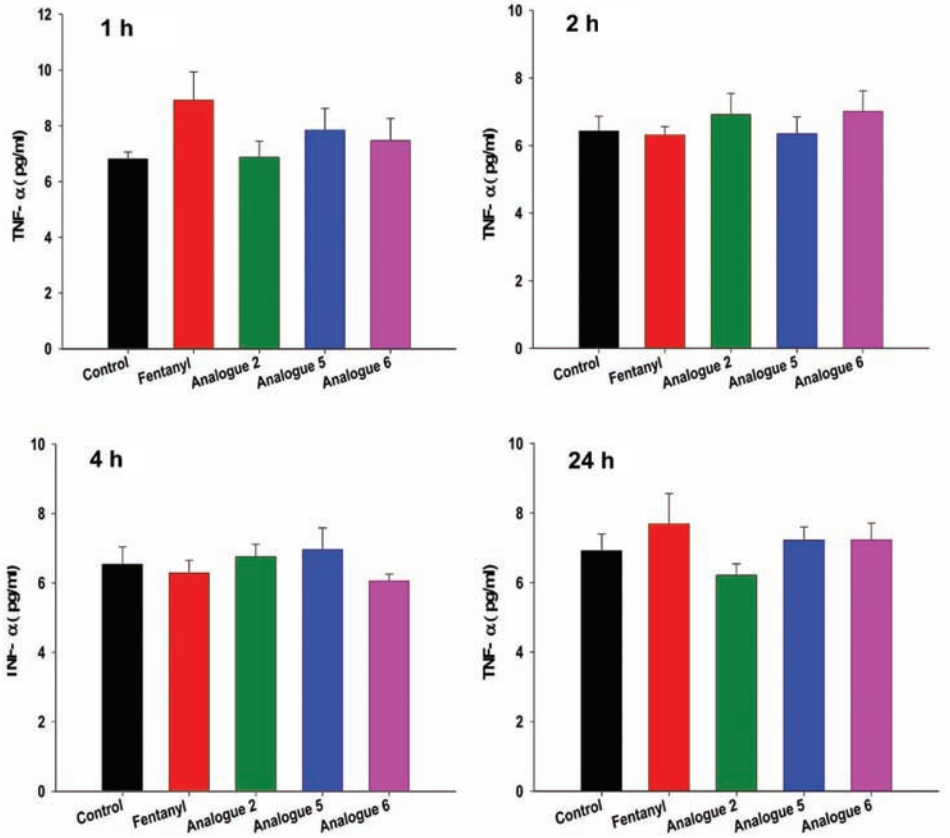


Figure 1. Male mice were intraperitoneally treated with fentanyl and its analogues 2, 5, and 6 (0.50 LD_{50}). Plasma levels of TNF-α were measured 1 h, 2 h, 4 h, and 24 h post- exposure. Values are mean ± SEM (n=6).

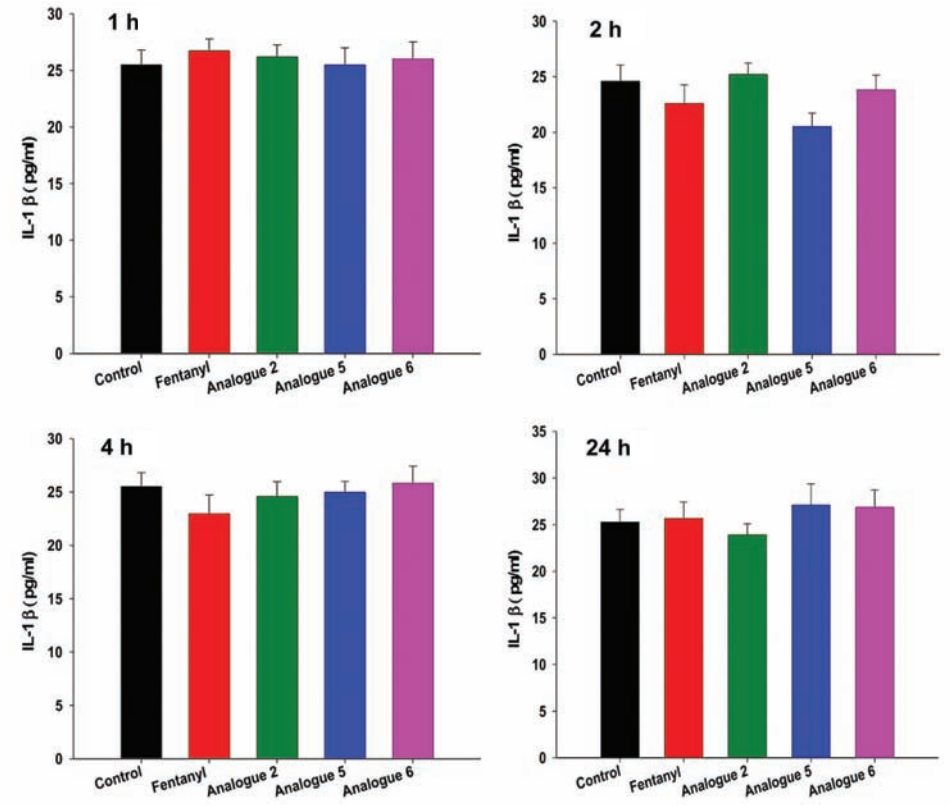


Figure 2. Male mice were intraperitoneally treated with fentanyl and its analogues 2, 5, and 6 (0.50 LD_{50}) . Plasma levels of IL-Iβ were measured 1 h, 2 h, 4 h, and 24 h post-exposure. Values are mean ± SEM (n=6).

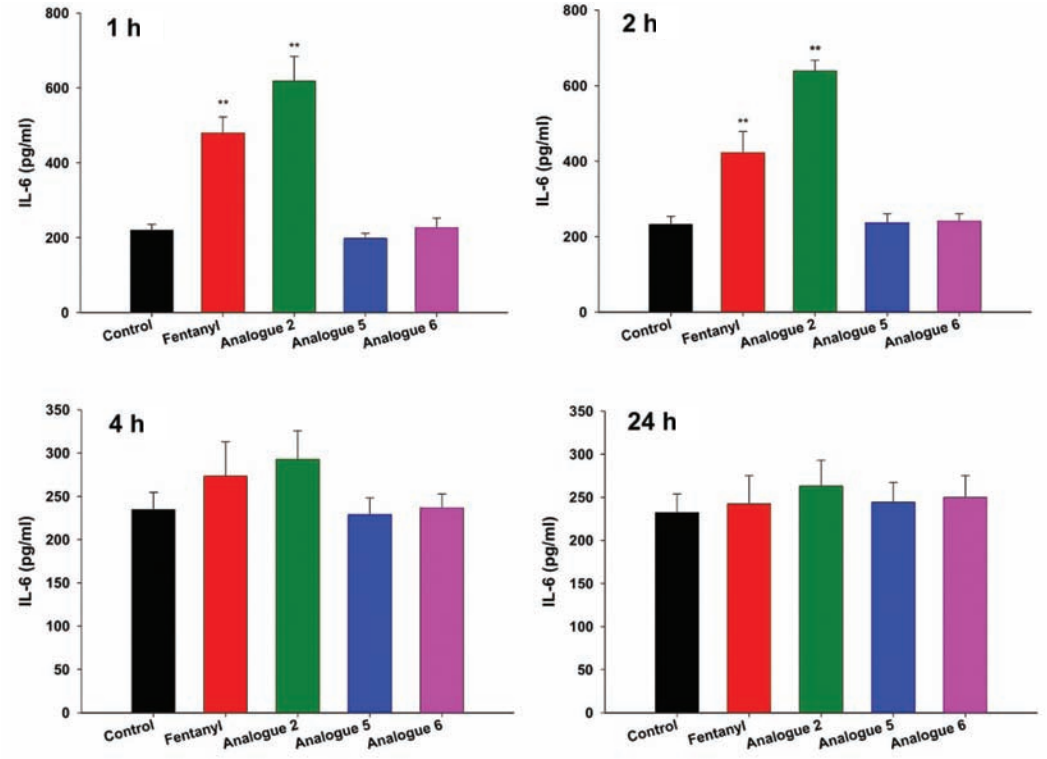


Figure 3. Male mice were intraperitoneally treated with fentanyl and its analogues 2, 5, and 6 (0.50 LD_{50}). Plasma levels of IL-6 were measured 1 h, 2 h, 4 h, and 24 h post-exposure. Values are mean ± SEM (n=6). Statistical significance was drawn at **p<0.01.

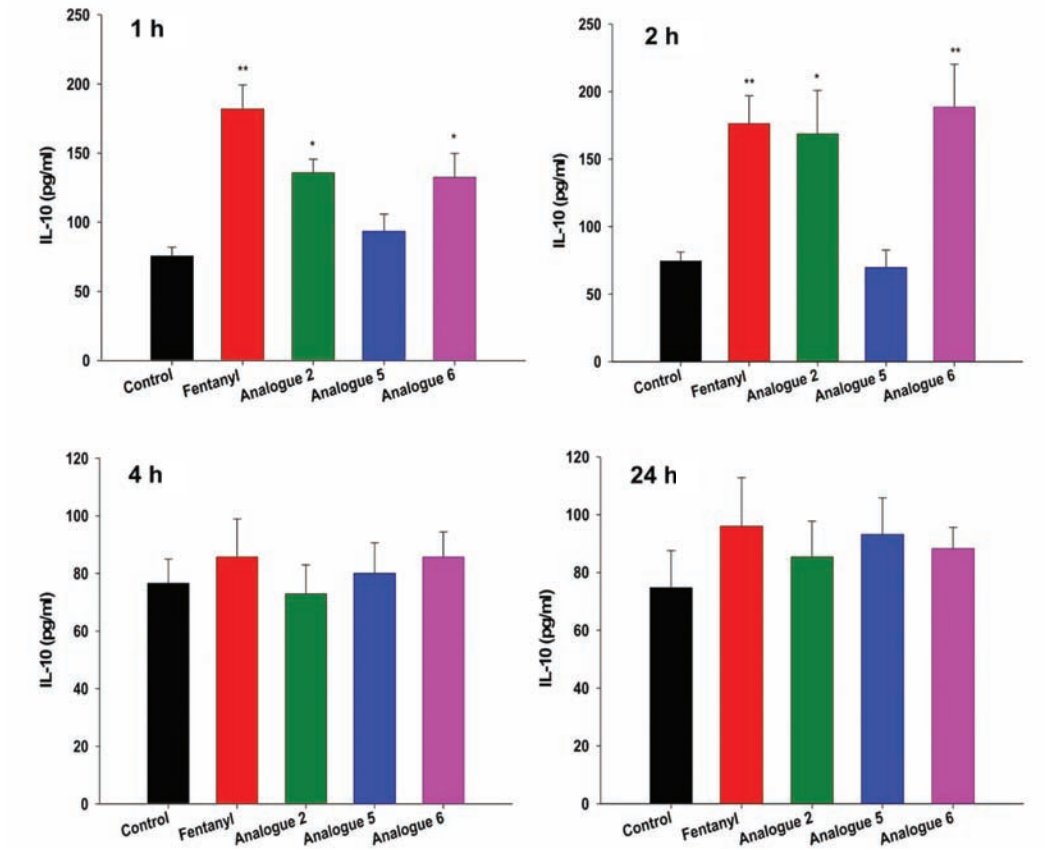


Figure 4. Male mice were intraperitoneally treated with fentanyl and its analogues 2, 5, and 6 (0.50 LD_{50}) . Plasma levels of IL- 10 were measured 1 h, 2 h, 4 h, and 24 h post-exposure. Values are mean ± SEM (n=6). Statistical significance was drawn at *p<0.05 and **p<0.01.

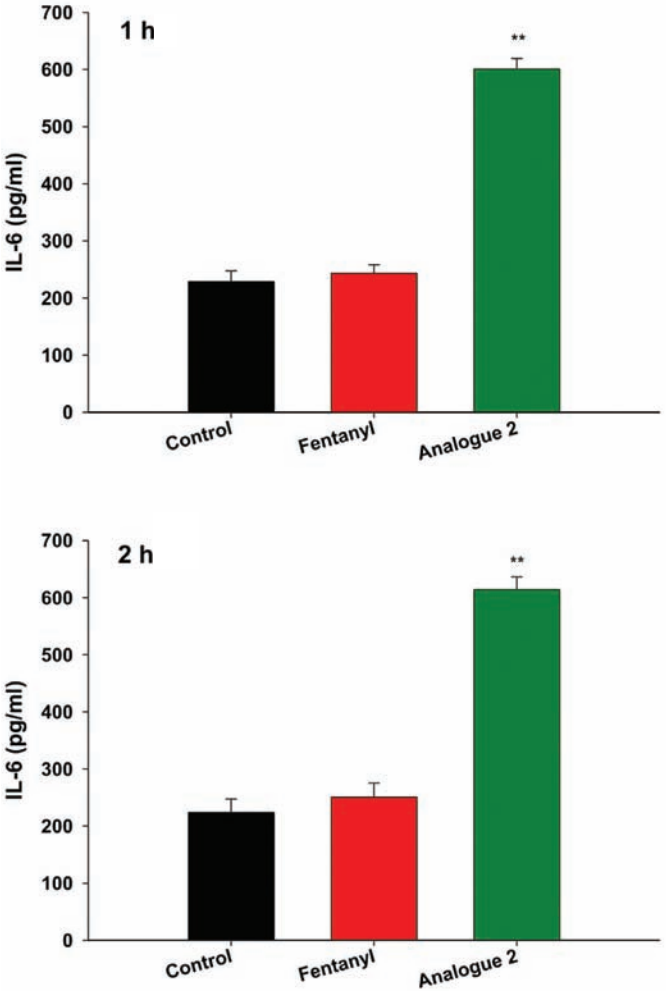


Figure 5. Male mice received pre-treatment (-30 min) of naltrexone hydrochloride (10 mg/kg; subcutaneous) followed by intraperitoneal injection of fentanyl or analogue 2 (0.50 LD_{50}). Plasma levels of IL-6 were measured 1 h and 2 h post-exposure. Values are mean ± SEM (n=6). Statistical significance was drawn at **p<0.01.

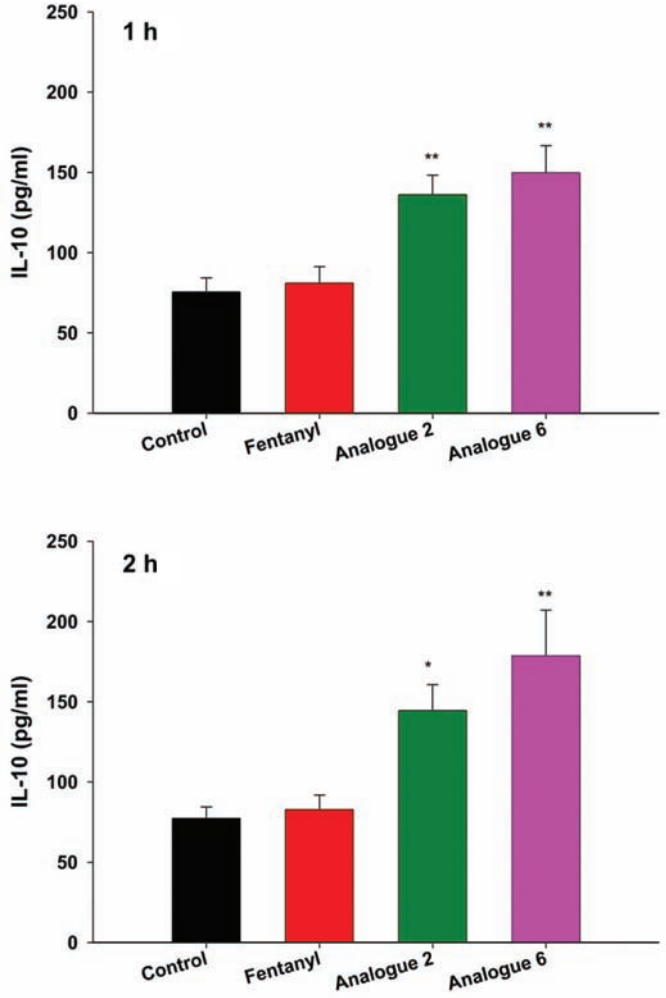


Figure 6. Male mice received pre-treatment (-30 min) of naltrexone hydrochloride (10 mg/kg; subcutaneous) followed by intraperitoneal injection of fentanyl or analogue 2 and 6 (0.50 LD_{50}). Plasma levels of IL-10 were measured 1 h and 2 h post-exposure. Values are mean ± SEM (n=6). Statistical significance was drawn at *p<0.05 and **p<0.01.

4. DISCUSSION

Opioids have a variety of effects on the immune system. Recently much attention has been emphasised on opioids and its impact on the immune system[13,14]. Opioids have been shown to modulate immune function by direct effects on cells of the immune system and indirectly via centrally mediated neuronal mechanisms[6]. In the present study, the immunomodulatory effect of fentanyl and its analogues was estimated in terms of cytokine levels in the blood plasma. In our present study, no significant change was observed in TNF-α and IL-1β levels in fentanyl and its analogues treated groups. In a previous study, incubation of whole blood in the presence of lipopolysaccharide (LPS) demonstrated a significant increase in TNF-α level in fentanyl treated group and, incubation of whole blood in the absence of LPS did not result in altered levels of TNF-α[7]. In this study, Wu[7], *et al.* demonstrated that fentanyl suppressed LPS-induced cytokines production. Moreover, they advocated that fentanyl modulates immune function directly through binding to the MOR present on cells of the immune system. IL-6 is produced by both immune and non-immune tissues and hence has numerous different biological effects. For instance, circulating IL-6 leads to the release of acute phase proteins from the liver[15], elicits a febrile response and activates the hypothalamic-pituitary-adrenal axis (HPA axis)[16]. Illicit or therapeutic use of opioids could lead to a dysregulation of circulating IL-6 levels. In our present study, IL-6 levels were increased in fentanyl and analogue **2** treated groups 1 h and 2 h post-exposure. Moreover, increases in IL-6 levels were more prominent in the analogue **2** treated group compared to fentanyl. Previous study also showed a significant increase in plasma IL-6 up to 2 h after morphine administration (10 mg/kg, s.c.) in rats[9]. It has been demonstrated that opioids can affect the cells of immune system directly through the opioid receptors on lymphocytes and macrophages[17]. Moreover, opiates can regulate the functions of the immune system through the nervous system[9,18]. It has been shown that increased production of IL-6 depends on the activity of the HPA axis and can be blocked by adrenalectomy[9].

In the present study, the anti-inflammatory cytokine IL-10 was found to significantly increase in fentanyl and analogue **2**, and **6** treated group up to 2 hr. Our results are in agreement with previous studies where a significant increase in IL-10 was observed in patients undergoing coronary artery bypass graft after sufentanil and fentanyl anesthesia[19]. However, in another study a significant decrease in IL-10 levels were observed in spleen cells after heroin administration[20]. There are conflicting reports on anti- or pro-inflammatory properties of μ-opioids[21]. For instance, morphine inhibits carrageenan-induced paw swelling[22] and was able to attenuate adjuvant arthritis in rats near toxic doses[23,24]. Conversely, low doses of morphine were pro-inflammatory in adjuvant arthritis[25]. Different opioids show different effects on the immune system such as immunostimulatory, immunosuppressive, or dual effect[26]. However, how they affect the different cytokines is not well defined[27]. Immunosuppressive activity depends on the type of the opioid, independent of the potency or the duration of action[28]. Moreover, Liang[26], *et al.* advocated that administration of short term/ low dose opioid has a positive impact and administration of long term/ high dose opioid has a negative impact on the immune system. In previous study, analogue **2, 5** and **6** were found to produce respiratory depression in a dose- dependent manner, which was similar to that caused by fentanyl[29].

In order to ascertain the involvement of opioid receptor, pre-treatment of naltrexone was used. We observed that in fentanyl treated group, elevation of plasma IL-6 and IL-10 levels was completely blocked by pre-treatment with naltrexone, thus demonstrating that this effect of fentanyl is opioid receptor-dependent. Here, our observations are in agreement with previous studies suggesting a role for opioid receptors in the elevation of serum IL-6 after morphine administration[9]. However, in analogue **2** treated group elevation of IL-6, and IL-10 and in analogue **6** treated group, elevation of IL-10 were not blocked by naltrexone. This indicates that immunomodulatory changes caused by analogue **2** and **6** could have some additional implications as well. The most probable reason for this is that exogenous opioids exert effects through multiple types of receptors[30]. Furthermore, Pacifici[30], *et al.* observed that central opioid or non-opioid receptors are involved in exogenous opioid-induced stimulatory effects, whereas peripheral opioid or non-opioid receptors are involved in depressive effects.

5. CONCLUSIONS

Opioid analgesics are known to exhibit diverse immunomodulatory properties. In our present study, fentanyl and its analogue **2** and **6** were found to significantly alter the levels of various cytokines at 0.50 LD_{50} (i.p.), which could be construed as undesirable effect in pain management. However, analogue **5** did not show any undesirable effects at the same dose.

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