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Nalbuphine, a kappa opioid receptor agonist and mu opioid receptor antagonist attenuates pruritus, decreases IL-31, and increases IL-10 in mice with contact dermatitis

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Abstract

Chronic itch is one of the disturbing symptoms of inflammatory skin diseases. Kappa opioid receptor agonists are effective in suppressing scratching in mice against different pruritogens. Nalbuphine, a nonscheduled kappa opioid receptor agonist and mu opioid receptor antagonist, has been in clinical use for post-operative pain management since the 1980s and recently has been in clinical trials for chronic itch of prurigo nodularis (https://www.trevitherapeutics.com/nalbuphine). We studied whether nalbuphine is effective against chronic scratching induced by rostral neck application of 1-fluoro-2,4-dinitrobenzene (DNFB), an accepted mouse model of contact dermatitis to study pruritoceptive itch. Mice were treated once a week with either saline or nalbuphine 20 min before the third, fifth, seventh, and ninth sensitizations with DNFB and the number of scratching bouts was counted for 30 min. Skin samples from the neck of mice at week 4 were used to measure protein levels and mRNA expressions of chemokines and cytokines. Different sets of mice were used to study sedation and anhedonic-like behavior of nalbuphine. We found that: nalbuphine (a) antagonized scratching in a dose- and time-dependent manner without affecting locomotion, b) decreased IL-31, and increased anti-inflammatory IL-10, and c) induced more elevations in the levels of CCL2, CCL3, CCL12, CXCL1, CXCL2, CXCL9, CXCL10, IL-1β IL-16, TIMP-1, M-CSF, TREM-1 and M1-type macrophages compared to saline. Increases in

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chemokines and cytokines and M1 macrophages by nalbuphine suggest an inflammatory phase of healing in damaged skin due to scratching. Our data indicate that nalbuphine is an effective antipruritic in murine model of pruritoceptive itch.

Keywords

Nalbuphine; pruritus; contact dermatitis; DNFB; cytokines; chemokines

1. Introduction

Chronic itch is one of the most disturbing symptoms encountered not only in some inflammatory skin diseases but also in systemic, neuropathic and psychological disorders (Yosipovitch and Bernhard, 2013; Xu et al., 2016). Treatment of chronic pruritus requires a wide range of systemic medications including antihistamines, glucocorticoids, antidepressants, gabapentin, methotrexate, cyclosporine, capsaicin, and calcineurin inhibitors (Pereira and Stander, 2017; Giavina-Bianchi and Giavina-Bianchi, 2019). Clinical studies targeting neurokinin 1, opioid, histamine, leukotriene, interleukin and TRPV1 receptors, and inhibitors on janus kinase and bile acid transporters are underway (Erickson et al., 2018; McEwen et al., 2018; Patel and Dao, 2018; Fowler and Yosipovitch, 2019).

The therapeutic value of kappa opioid receptor agonists against chronic pruritus has increased. Previous studies have shown that several kappa opioid receptor antagonists induce compulsive scratching (Kamei and Nagase, 2001; Inan et al., 2011; Cowan et al., 2015) whereas kappa opioid receptor agonists suppress scratching in mouse models of acute (Togashi et al., 2002; Ko et al., 2003; Inan and Cowan, 2004; Inan et al., 2009; Sakakihara et al., 2016), and chronic itch (Umeuchi et al., 2005; Inan and Cowan 2006; Akiyama et al., 2015; Takahashi et al., 2017). Further, dynorphin, an endogenous kappa opioid receptor agonist, plays a neuromodulatory role in the conduction of itch stimuli along the spinal cord (Kardon et al., 2014). Recent preclinical studies have focused on the ability of kappa opioid receptor agonists to signal preferentially via G proteins rather than β-arrestin 2 (thus causing fewer side effects) in targeting pruritus and pain (Brust et al., 2016).

Nalfurafine, the kappa opioid receptor agonist, has been approved for the treatment of uremic pruritus and is in clinical trials for pruritus in chronic liver disease in Japan (Kamimura et al., 2017). Difelikefalin and asimadoline, peripherally acting kappa opioid receptor agonists, are in clinical trials for uremic pruritus and atopic dermatitis, respectively (Chalmers, 2011; McEwen et al., 2018). Promising results have been reported with nalbuphine, a mu opioid receptor antagonist and kappa opioid receptor agonist, against prurigo nodularis (https://www.trevitherapeutics.com/nalbuphine). Nalbuphine has been in clinical practice since the 1980s for postoperative pain management (Beaver and Feise, 1978; Beaver and Feise 1981), and since the 1990s for reversal of morphine-induced pruritus without affecting analgesia suggesting an action through kappa opioid receptor agonism (Alhashemi et al., 1997; Yeh et al., 2009; Moustafa et al., 2016).

The role of the immune system in itch in mice and humans with inflammatory skin diseases has been reported (Dillon et al., 2004; Bilsborough et al., 2006; Takamori et al., 2018;

Hashimoto et al., 2019). The present study investigated nalbuphine's effect on pruritus and chemokine and cytokine levels in a DNFB-induced contact dermatitis, a mouse model of pruritoceptive itch (Zhang et al., 2015; Vaia et al., 2016; Mu et al., 2017; Mack and Kim, 2018). The presence of regulatory T (Treg) cells, and M1 and M2 monocytes/macrophages were also examined. Additionally, sedation and anhedonic-like effects were studied as recognized side effects of nalbuphine.

2. Materials and methods

2.1. Animals

Male Swiss Webster mice (Taconic Biosciences, Germantown, NY) weighing 25-30 g were used. Mice were housed in a temperature- and humidity-controlled environment with a 12-hr light-dark cycle. They were supplied with food and water *ad libitum*. Before any procedure was applied, the mice were acclimated for 1 week in the animal facility. Behavioral testing was performed between 11:00 AM and 5:00 PM. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Temple University and conducted according to the NIH Guide for the Care and Use of Laboratory Animals. In all experiments, 6-8 mice/group were used.

2.2. Acute administration of nalbuphine against DNFB-induced scratching

We used a model of contact allergic dermatitis induced by repeated exposure to DNFB-acetone solution (Zhang et al., 2015, Mu et al., 2017). Two days before DNFB application, the rostral area of the neck and the abdomen of mice were shaved. Initial sensitization with DNFB-acetone solution (100 μl) was conducted on abdominal skin. One week later, a cutaneous reaction was evoked on a rostral area of the neck by repeated (2 times a week for 4 weeks) applications of 50 μl of DNFB-acetone solution. Nine applications were made, including initial sensitization. The timeline and sequence for these experiments are shown in Fig. 1. Mice were randomly divided into the following four groups: saline + DNFB, nalbuphine 1 mg/kg + DNFB, nalbuphine 3 mg/kg + DNFB, and nalbuphine 10 mg/kg + DNFB. Every week (for 4 weeks) before the second sensitization, mice were placed into observation boxes for at least for an hr for acclimation. Twenty min before application of the DNFB solution, mice were injected with either saline or nalbuphine (1, 3, or 10 mg/kg, s.c.). The DNFB-acetone solution was then applied to the rostral area of neck and 1 min later, the number of scratching bouts directed to the neck was counted for 30 min. At the end of observations on week 4, the mice were euthanized using CO_2 followed by cervical dislocation. Neck skin tissue and abdominal skin tissue (as control) were obtained from mice treated with saline or nalbuphine (10 mg/kg) and kept at −80 °C to measure chemokine and cytokine levels.

2.3. Measurement of locomotion and anhedonia-like effect

In a different group of mice (n=8), we examined whether the anti-scratch effect of nalbuphine is a result of behavioral depression and/or anhedonic-like behavior since sedation and anhedonia are known side effects of kappa opioid agonists. For locomotor activity, mice were placed individually in a cage (27 cm x 48 cm x 20 cm) and acclimated for 1 hr. They were injected s.c. with either saline or nalbuphine (10 mg/kg) and monitored for total

distance traveled over 1 hr using a Digiscan D Micro System (AccuScan, Columbus, OH). We chose the highest dose of nalbuphine since the sedation effect is dose-related.

The possible anhedonic-like effect was measured using the splash test which quantitates self-grooming (Yalcin et al., 2008; Butelman et al., 2019). Mice were acclimated individually to observation cages for at least 1 hr and were then injected with either saline or nalbuphine (3 or 10 mg/kg, s.c.). Twenty min later, using a spray bottle, approximately 0.7 ml of 10 % sucrose (w/v) solution in water was sprayed from about 10 cm distance on the flank areas of the mouse. The time spent grooming on both flank areas was recorded for 5 min and for 10 min (in different mice) using a stopwatch.

2.4. Measurement of chemokine and cytokine levels

Chemokine and cytokine levels (except IL-31) were measured in neck tissue lysates using a proteome profiler mouse cytokine array kit purchased from R&D Systems Inc. (Minneapolis, MN). The relative expression levels of 40 mouse cytokines were detected using nitrocellulose membranes that have antibodies spotted in duplicate. Neck tissue lysates were prepared as follows: tissues were homogenized in phosphate-buffered saline with protease inhibitors. After homogenization, Triton X-100 was added (final concentration was 1% in homogenate). Samples were frozen at −80 °C, thawed, and then centrifuged at 10,000 x g for 5 min to remove cellular debris. Homogenates were kept at −80 °C. Protein concentration of samples was measured using a Thermo Fisher Scientific NanoDrop 2000 spectrophotometer. The proteome profiler array kit had 4 nitrocellulose membranes loaded with the same antibodies. Each membrane was used for one group. For each group, cytokine and chemokine levels were measured in pooled samples (6-8 mice). 300 μg samples for each group were used. Sample/antibody/streptavidine-HRP complex was measured using chemiluminescent detection reagents. Light is produced at each spot in proportion to the amount of cytokine bound. Luminescence was quantitated by a Fuji Digital camera using imageGauge® software. Results were stated as Arbitrary Units and reported as fold change from control (abdominal skin results for saline + DNFB group).

IL-31 was measured using a Mouse IL-31 PicoKine ELISA kit (Boster Biological Technology Co., Pleasanton, CA) in duplicate homogenized neck skin tissue samples from mice treated with saline and nalbuphine (10 mg/kg) according to the manufacturer’s guidelines.

2.5. Measurement of mRNA expression

Homogenized neck tissue samples from mice treated with saline and nalbuphine (10 mg/kg) were used. Analyses of RNA expression were performed as previously described (Sanmiguel et al., 2009). Briefly, RNA was isolated using a RNeasy kit (Qiagen) and cDNA synthesized with AMV Reverse Transcriptase (Promega) and random hexamers. Real time PCR was carried out using a StepOnePlus Real-Time PCR System (Applied Biosystems) and the primers listed below. FoxP3 (Treg), iNOS (M1 monocyte/macrophage), CD80 and CD163 (M2 monocyte/macrophage) markers were used. We also measured mRNA expression for IL-10 since it is more sensitive than protein measurement. Relative expression of every gene was normalized to GAPDH by the 2ΔCT method. The following primers were used:

GAPDH (F) CTTGTGCAGTGCCAGCC (R) GCCCAATACGGCCAAATC;

FoxP3 (F) CTCCCTGCTCCTCCTATTC (R) CTCCTAATGCCTCCCAGAG;

IL-10 (F) GCCCTTTGCTATGGTGTC (R) TCCCTGGTTTCTCTTCCC;

iNOS (F) CAGCTACGCCTTCAACAC (R) TGGGACAGTCTCCATTCC;

CD80 (F) CTCGCTTCTCTTGGTTGG (R) TGGTTGCGAGTCGTATTG;

CD163 (F) ACTCCAGGAAGGGCATAC (R) CACAGCCCAACTGCTTAC

2.6. Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.) and $P<0.05$ was accepted as statistically significant. GraphPad Prism, version 7, was used for data analysis. To analyze results for the effects of nalbuphine on pruritus induced by DNFB, two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used. IL-31 levels, IL-10, FoxP3, iNOS, CD80, CD163, and locomotion were analyzed using unpaired Student's t-test. Splash test results were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Statistics on the results of the RT2 Profiler® Arrays were done by the Student's t-test, using the online GeneGlobe Data Analysis Center (Qiagen).

2.7. Compounds

1-Fluoro-2,4-dinitrobenzene and nalbuphine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). DNFB 0.1% solution was prepared in acetone and nalbuphine was dissolved in saline.

3. Results

3.1. Acute systemic administration of nalbuphine attenuates DNFB-induced pruritus

DNFB sensitization induced scratching beginning during the first week and continuing until the end of observations on week 4 (Fig. 2). Nalbuphine at 3 and 10 mg/kg given 20 min before the fifth (week 2), seventh (week 3), and ninth (week 4) DNFB challenges significantly decreased scratching (*$P<0.05$, **$P<0.01$, ****$P<0.0001$). At the third sensitization (week 1), there was a decrease in scratching with nalbuphine 10 mg/kg pretreatment, however it did not reach a statistically significance level. Nalbuphine antagonized scratching in a time- and dose-dependent manner.

3.2. The effect of nalbuphine on locomotion and anhedonia

To establish if the antagonizing effect of nalbuphine on pruritus is a consequence of sedation, we measured locomotion in mice treated with saline or nalbuphine 10 mg/kg. Ambulation was not significantly suppressed in mice treated with nalbuphine compared to saline administered mice (Fig. 3).

Anhedonic-like behavior was tested using the splash test that measures self-grooming. We measured grooming for 5 min and 10 min durations in different groups of mice (Fig. 4). Nalbuphine (3 and 10 mg/kg) significantly reduced grooming time by 70-75% compared to saline during the 5 min observation period (Panel A, **P<0.01). In mice observed through 10 min, both 3 and 10 mg/kg of nalbuphine significantly decreased grooming by approximately 20% compared to saline (Panel B, *P<0.05).

3.3. Changes in chemokine and cytokine levels

To examine if nalbuphine would influence inflammation in dermatitis, we used an array that measures a wide spectrum of chemokines and cytokines. Array images from abdominal and neck tissues of mice treated with saline or nalbuphine (10 mg/kg) are shown in Fig. 5. Abdominal skin of mice treated with nalbuphine did not show any detectable increase in chemokine expression from that of mice treated with saline. Increases were only noted on neck skin where DNFB was applied. This clearly shows that chemokine levels were increased as a consequence of dermatitis. Fold changes in chemokine (A) and cytokine (B) levels in neck tissue of saline + DNFB, abdominal tissue of nalbuphine + DNFB, and neck tissue of nalbuphine + DNFB compared to abdominal tissue of saline + DNFB are summarized in Table 1. CCL2, CCL3, CCL12, C5a, CXCL1, CXCL2, CXCL9, CXCL10 levels were increased 16-, 13-, 4-, 7-, 4-, 10-, 7-, and 4-fold, respectively, in mice with dermatitis treated with saline. However, the same chemokines were increased to a greater extent in nalbuphine-treated mice with dermatitis (60-, 57-, 10-, 13-, 8-, 80-, 14-, and 7-fold, respectively). While CCL1, CCL5, and CXCL11 did not show any change on neck tissue of mice treated with saline, they were slightly increased in nalbuphine-treated animals (4-, 4-, and 3-fold, respectively). CCL4, CXCL12, and CXCL13 levels did not change in either saline- or nalbuphine-treated mice with dermatitis. For cytokines, abdominal skin also did not show any increase except for IL-16 (6-fold). IL1-β, IL-13, IL-16, TIMP-1, M-CSF, TREM-1, SICAM, and IFN-γ levels were increased 4-, 5-, 10-, 6-, 4-, 4-, 5-, and 3-fold, respectively, in mice with dermatitis treated with saline. Similar to chemokines, these cytokines (except IL-13, SICAM, and IFN-δ) were increased more in mice with dermatitis treated with nalbuphine (16-, 3-, 16-, 12-, 10-, 25-, 4-, and 3-fold, respectively). While IL-1ra, IL-12p7c, IL-17, IL-23, and TNF-α did not show any changes in mice with dermatitis injected with saline, they were increased slightly in nalbuphine-injected mice (4-, 3-, 3-, 3-, and 4-fold, respectively). IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-27, G-CSF, and GM-CSF levels did not change in either saline- or nalbuphine-treated mice with dermatitis (Table 1.). Nalbuphine has an effect on the release of chemokines and cytokines in skin with dermatitis.

3.4. Nalbuphine decreases IL-31 level and increases IL-10 mRNA expression

Previous studies have reported that IL-31, a cytokine for type 2 immune responses, plays an important role for inducing pruritus and inflammation in atopic dermatitis and chronic contact dermatitis in mice and humans (Dillon et al., 2004; Bilsborough et al., 2006; Sonkoly et al., 2006; Cevikbas et al., 2014; Takamori et al., 2018). To study if nalbuphine has an effect on IL-31, we measured IL-31 levels in mice treated with saline or nalbuphine (10 mg/kg) using ELISA. The IL-31 level was significantly decreased in nalbuphine-administered mice compared to saline-injected mice (Fig. 6 Panel A, *P<0.05).

IL-10 is a Type 2 anti-inflammatory cytokine that downregulates expressions of Type 1 cytokines, IFN-γ, IL-2, IL-3, TNF-α, GM-CSF, and IL-31. When IL-10 was given to patients with psoriasis their lesions were decreased (Couper et. al., 2008; Mosser and Zhang, 2008; Shachar and Karin, 2013). We found IL-10 protein levels to be below the detection limit of the arrays (Fig. 5); therefore, we examined IL-10 mRNA expression, since it is more sensitive than protein measurements. IL-10 mRNA expression was significantly increased in mice treated with nalbuphine compared to mice treated with saline (Fig. 6 Panel B, *P<0.05). Nalbuphine decreased pruritic IL-31 and increased anti-inflammatory IL-10. Since IL-10 downregulates IL-31, it is suggested that nalbuphine decreases IL-31 by increasing IL-10.

3.5. Nalbuphine increases M1 monocyte/macrophage response

Given the chemokine and cytokine results with nalbuphine (Table 1.), we next measured mRNA expression of FoxP3 (Treg), iNOS (M1 monocyte/macrophage), and CD80 and CD163 (M2 monocyte/macrophage) to identify the type of immune cells present in skin with dermatitis. Expression for FoxP3 mRNA was similar in mice treated with saline or nalbuphine (Fig. 7 Panel A). Nalbuphine significantly increased iNOS mRNA expression compared to saline (Fig. 7 Panel B, *P<0.05). Both CD80 and CD163 mRNA expressions did not show any difference in mice injected with saline or nalbuphine (Fig. 7 Panel C and D, respectively). Nalbuphine increased Type 1 monocyte/macrophages in skin with dermatitis.

4. Discussion

We investigated if nalbuphine would be effective against pruritoceptive pruritus in chronic and inflammatory skin diseases like contact dermatitis. We used the DNFB-induced dermatitis mouse model to study the effect of nalbuphine on scratching as well as on chemokine and cytokine levels since it is an inflammatory situation. Further, we studied sedation and anhedonia-like behavioral side effects of nalbuphine. Major findings of this study are: 1) Nalbuphine, a mixed kappa opioid receptor agonist and mu opioid receptor antagonist, suppresses pruritus in the DNFB-induced mouse model of chronic contact dermatitis 2) Nalbuphine increases anti-inflammatory cytokine IL-10, and reduces pro-inflammatory IL-31 levels in the skin with dermatitis, and 3) Nalbuphine increases M1-type monocyte/macrophage as well as some chemokines that are possibly involved in the inflammatory phase of the healing process. These data suggest that nalbuphine promotes skin healing. Our study reports for the first time that there are changes in the release of a wide range of chemokines and cytokines due to nalbuphine treatment in pruritus.

Previously, anti-scratch activity of nalbuphine (10-30 mg/kg) was reported against Substance P-induced scratching in mice (Hawi et al., 2013), however, to our knowledge, the present study is the first to report that nalbuphine (3 and 10 mg/kg) is antipruritic in a mouse model of chronic contact dermatitis (a pruritoceptive pruritus) without inducing sedation with a high dose of nalbuphine. Recently, we have also reported that nalbuphine suppresses TAT-HIV-1 (trans activator of transcription)-, chloroquine- (Inan et al., 2019), and deoxycholic acid (a secondary bile acid)-induced (Cowan et al., 2019) scratching in mice. Itch is a

common symptom in patients with HIV. Chronic itch can be due to skin diseases that develop in almost 90% of HIV-positive patients or idiopathic (6%) (Gelfand and Rudikoff, 2001; Zancanaro et al., 2006; Kaushik et al., 2014; Parker, 2014). Prevalence of chronic pruritus in HIV-positive patients has been reported as 31% from a study conducted in Spain and 45% from a study conducted in southeastern United States (Blanes et al., 2012; Kaushik et al., 2014). Quality of life has been found negatively affected in HIV-patients with chronic itch in these studies. Cholestatic itch is an ongoing challenge in medicine that is often refractory to available therapeutics. Bile acids are believed to cause the repetitive scratching behavior by activating MRGPRX4 receptors in mice (Meixiong et al., 2019). Together all these results indicate that nalbuphine is a widely effective suppressor of scratching (in mice).

Also, nalbuphine at 3 and 10 mg/kg decreased only 20-25% grooming during a 10 min observation. Nalbuphine has advantages of over other candidate kappa opioid receptor agonists. First, it has been in clinical practice since the 1980s; second, it is not a scheduled compound in the USA, for example nalfurafine is scheduled as class II; and third, in clinical studies it has been shown to induce fewer psychotomimetic episodes compared to other kappa opioid agonists such as pentazocine and butorphanol (Schmidt et al., 1985).

Takamori et al. (2018) reported that IL-31-deficient mice develop contact dermatitis with less scratching to DNFB-induced sensitization. They suggested that IL-31 is required for the induction of pruritus. In our study, nalbuphine significantly reduced IL-31 levels (Fig. 6A) and scratching (Fig.2) also suggesting that IL-31 plays an important role in the development of pruritus. Further, nalbuphine significantly increased IL-10, an anti-inflammatory cytokine (Fig. 6B). IL-10 downregulates expression of Th1 cytokines like IL-2 and interferon-γ (IFN-γ), limits pro-inflammatory cytokine release (Tumor Necrosis Factor-α (TNF-α), IL-1, IL-6, IL-12), and controls differentiation of macrophages, T and B cells (Couper et al., 2008; Shachar and Karin, 2013). The anti-inflammatory effect of kappa opioid agonists has been reported in inflammatory pain models in rats (Walker et al., 1995; Binder et al., 2001; Walker 2003; Bileviciute-Ljungar et al. 2006) and in mice (Paton et al., 2017). Also, Bastos et al. (2011) showed that U-50,488 (a kappa opioid receptor agonist) reduced bone loss and IL-6 levels and increased IL-10 in ligature-induced periodontal disease in rats.

In our study, mice treated with saline had major increased levels of CCL2, CCL3, CXCL2, IL-1β, IL-13, and IL-16 (Table 1.). There was no increase in IL-4, IL-5 and IL-9 that are involved in the type 2 immune response. Only IL-13 was increased in terms of type 2 response. Pro-inflammatory chemokines CCL2, CCL3, CXCL2 and cytokines IL-β, and IL-16 were increased. Nalbuphine induced further increases in CCL2, CCL3, CXCL2, IL-1β, and IL-16 in addition to increases in CXCL9, CXCL10, Macrophage Colony-Stimulating Factor (M-CSF), Metalloproteinase Inhibitor 1 (TIMP-1) and Triggering Receptor Expressed on Myeloid cells-1 (TREM-1) (Table 1.). Previous studies have shown that chemokines are involved in wound healing. It was shown that CCL3 (DiPietro et. al., 1998) and CCL2 (Wood et. al., 2014) are required for wound healing as chemoattractants for macrophages. During the inflammation phase of wound healing polymorphonuclear neutrophils and monocytes (differentiate to macrophages) come to the site. As the wound heals, Ml pro-inflammatory macrophages are replaced by M2 anti-inflammatory macrophages (Rees et al., 2015; Krzyszczyk et al., 2018). M1 macrophages secrete TNF-α,

IL-1β and IL-6 for a strong inflammatory response. CXCL2, CXCL10 and CXCL11 are important chemokines for wound healing, especially, CXCL10 and 11 initiating the remodeling phase (Behm et al., 2012; Brubaker et al., 2013; Rees et al., 2015). Nalbuphine treatment increased CCL2, CCL3, CXCL2, IL-β, CXCL9 and CXCL10 (Table 1.) as well as M1 type macrophages (Fig. 7) compared to saline administration. Further, M-CSF was also increased by nalbuphine (Table 1.). The positive role of M-CSF on wound healing has been reported previously (Ikeda et al., 2008; Sugiyama et al., 2008; Li et al., 2016). TIMP-1, also increased by nalbuphine (Table 1.), is a member of matrix metalloproteinases inhibitors that degrade the extracellular matrix, as well as being involved in cell growth and differentiation, cell migration, anti-angiogenesis, anti- and pro-apoptosis, and synaptic plasticity (Brew and Nagase, 2010). Positive effects of TIMP-1 in wound healing have also been reported (Arndt et al., 2018; Trestrup et al., 2018). It has been shown that TREM-1 plays a critical role in the development of inflammation in atherosclerosis and colitis (Boufenzer et al., 2015; Che et al., 2018). However, a recent study has reported that an increase in TREM-1 correlates with faster wound healing in cutaneous leishmaniasis (Nunes et al., 2018). Our results with nalbuphine suggest that skin with dermatitis collected on week 4 might reveal the beginning phase of wound healing. Further studies are required to answer the following questions: 1) What would the results be at weeks 5 or 6 in terms of chemokines and cytokines?, 2) What would the results be if nalbuphine was given chronically?, and 3) What would the histologic evidence be following chronic administration of nalbuphine?

In conclusion, this study demonstrates that nalbuphine is effective against pruritoceptive pruritus in dermatitis in mice. It decreases IL-31 and increases anti-inflammatory IL-10. Increase in some chemokines and cytokines as well as M1-type monocyte/macrophages by nalbuphine may suggest that nalbuphine promotes healing of inflamed skin. Collectively, in addition to suppression of scratching, this outcome would be beneficial.

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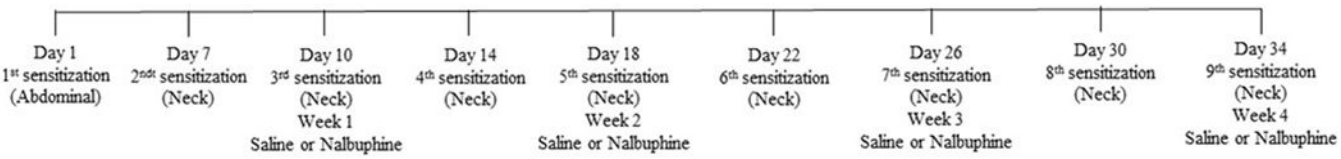

Fig. 1. Timeline for DNFB sensitizations and treatments.

Mice were sensitized twice a week (one week after first sensitization on abdominal skin) by rostral neck application of DNFB (0.1% solution in acetone). Twenty min before the third, fifth, seventh and ninth applications mice were treated (s.c.) with either saline or nalbuphine (1, 3, or 10 mg/kg). Following application of DNFB, the number of scratching bouts to the neck was counted for 30 min.

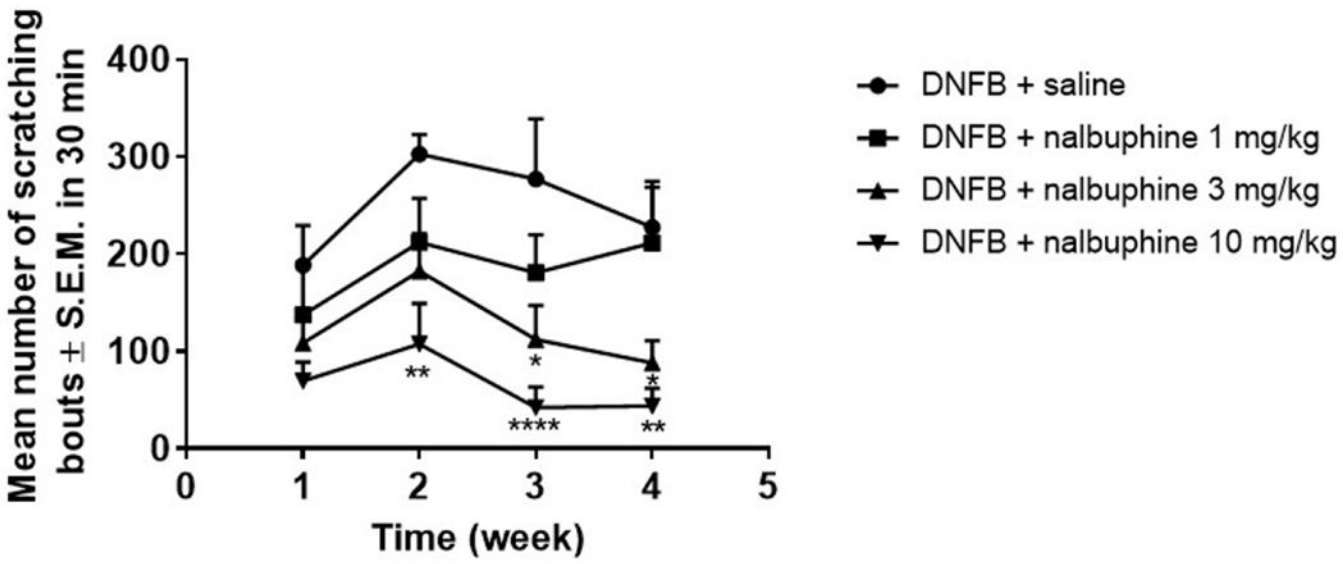

Fig. 2. Nalbuphine inhibits scratching in a time- and dose-dependent manner.

Nalbuphine at 3 and 10 mg/kg significantly reduced scratching compared to saline (Two-way ANOVA (time and treatment) followed by Dunnett's multiple comparison test, *P<0.05, **P<0.01, ****P<0.0001; n=6-8).

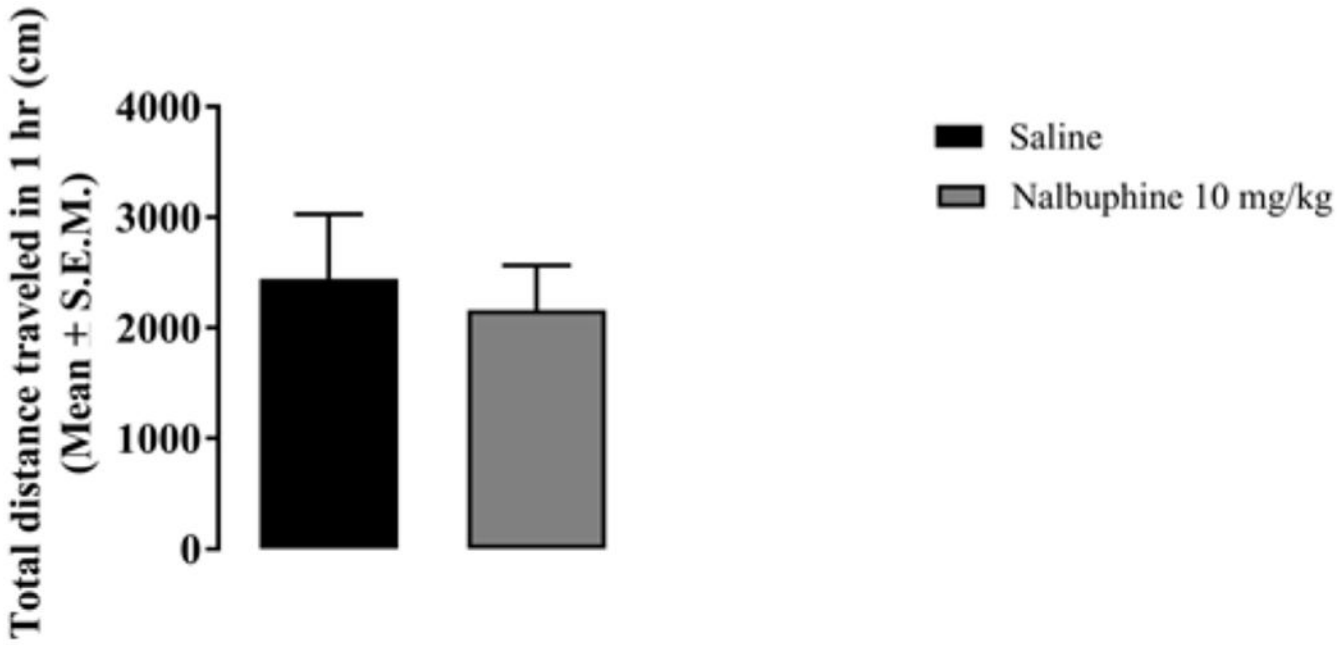

Fig. 3. Nalbuphine does not significantly affect locomotion.

Mice (n=8) were injected with either saline or nalbuphine (10 mg/kg) and then total distance traveled was measured for 1 hr. Ambulatory activity was similar in mice treated with saline and nalbuphine.

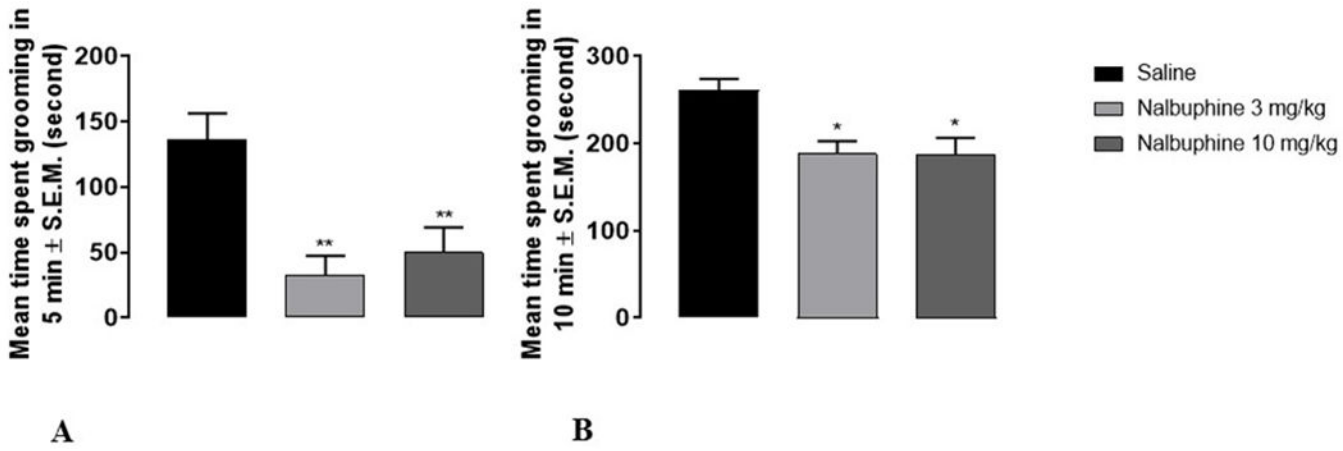

Fig. 4. Measurement of anhedonic-like effect of nalbuphine.

Mice were acclimated individually to observation boxes. They were injected (s.c.) with either saline or nalbuphine (3, or 10 mg/kg) 20 min before spraying 10% sucrose solution in water. Following spraying sucrose solution to the flank areas of mice, the time to spent grooming the flank areas was recorded during 5 min and 10 min (different group of mice). Both 3 and 10 mg/kg nalbuphine significantly reduced grooming time 70-75% compared to saline during the 5 min observation period (Panel A, **P<0.01,). In mice observed through 10 min, both 3 and 10 mg/kg nalbuphine significantly decreased grooming about 20% compared to saline (Panel B, *P<0.05). (One-way ANOVA followed by Dunnett's test, n=6-8).

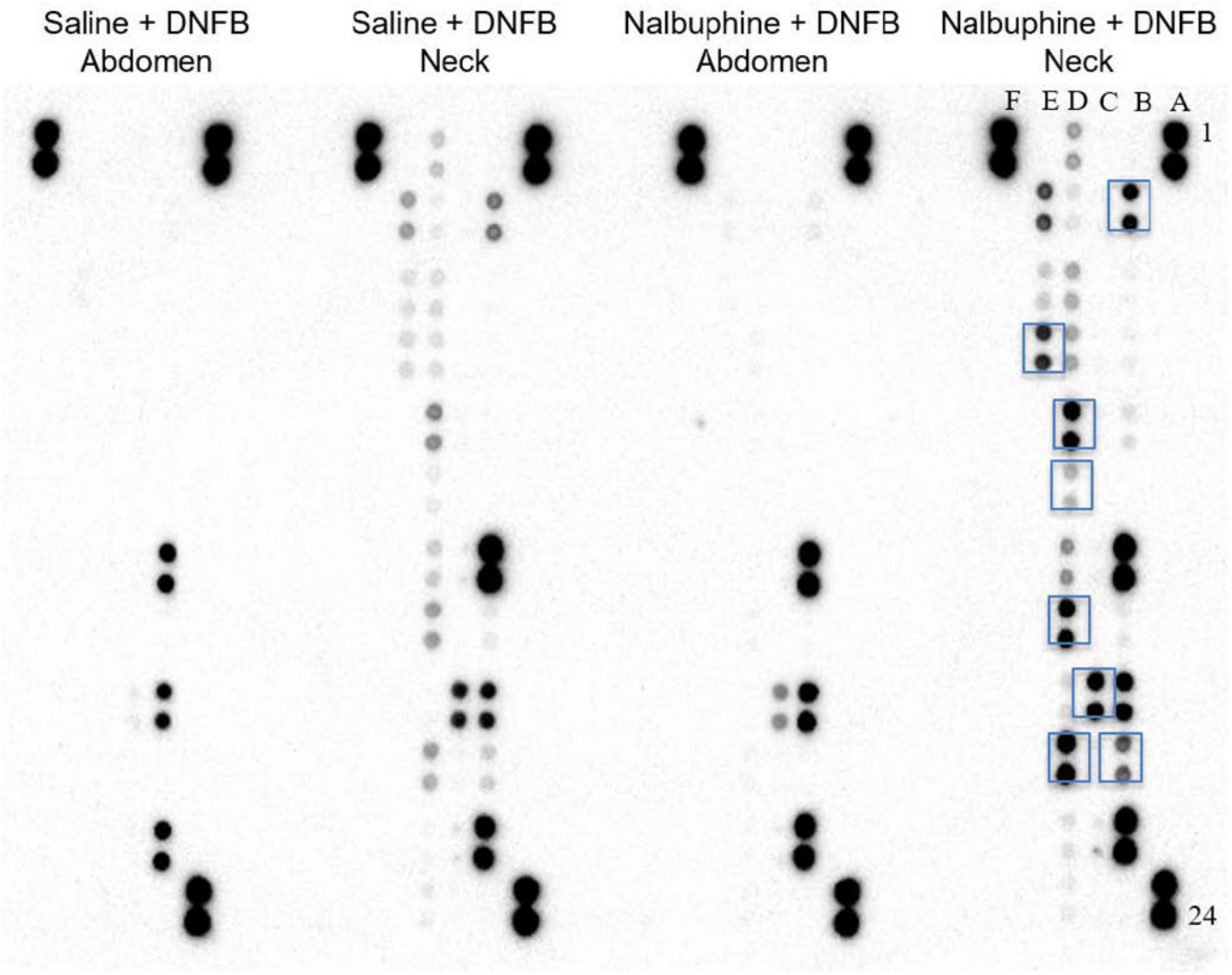

Fig. 5. Chemokine and cytokine levels were measured using proteome profiler array.

Nitrocellulose membranes are shown for abdominal and neck tissue for saline and nalbuphine (10 mg/kg) treatment. A1, 2, 23, 24, F1, 2 are reference spots, and F23, 24 are negative control. Boxed points on neck tissue sample treated with nalbuphine represent chemokines and cytokines that have increased to a greater extent compared to saline treatment: B3, 4 (CCL5); B19, 20 (IL-1β); C17, 18 (IL-16); D7, 8 (M-CSF); D9, 10 (CCL2); D15, 16 (CCL3); D19, 20 (CXCL2); E3, 4 (TIMP-1); E7, 8 (TREM-1).

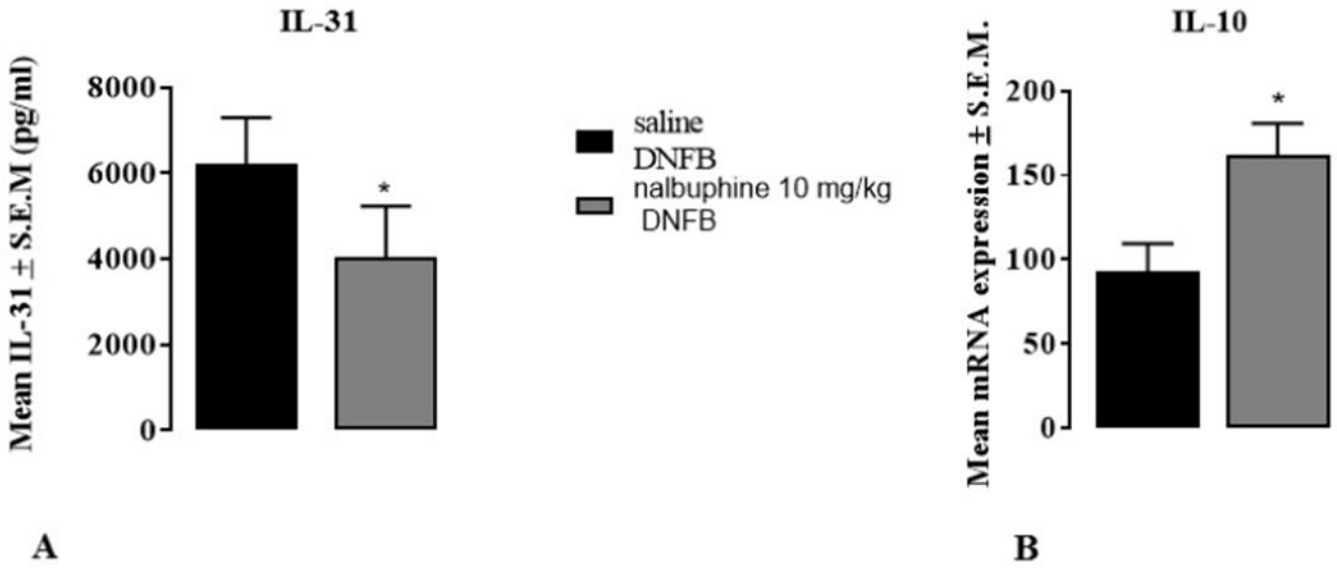

Fig. 6. Nalbuphine decreases IL-31 and increases IL-10.

A. Nalbuphine (10 mg/kg) significantly reduces IL-31 levels compared to saline in neck tissue samples. The IL-31 value was measured using ELISA. **B**. Nalbuphine (10 mg/kg) significantly increases IL-10 mRNA expression compared to saline in neck tissue samples. (*P<0.05, unpaired Student's t-test, n=6-8).

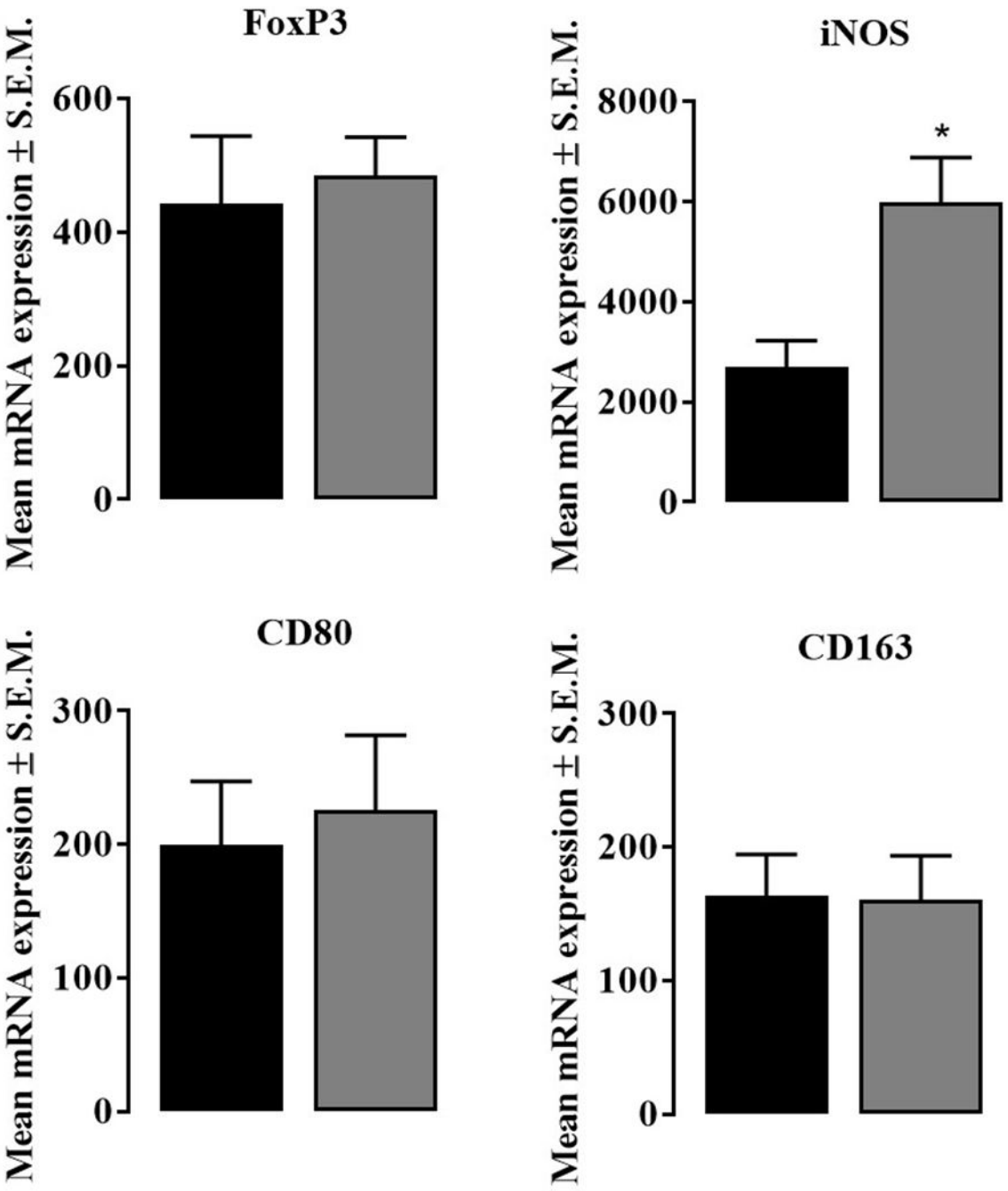

Fig. 7. mRNA expressions for markers for Treg, and M1 and M2 monocyte/macrophage.

FoxP3 (marker for T regular cells), iNOS (marker for M1 type monocyte/macrophage), CD80, and CD163 (markers for M2 type monocyte/macrophage). FoxP3, CD80, and CD163 mRNA expressions were similar in neck tissue samples from mice treated with saline and nalbuphine. iNOS mRNA expression was significantly increased in neck tissue samples from mice treated with nalbuphine (*P<0.05, unpaired Student's t-test, n=6).

Table. 1.

Fold changes in chemokine (A) and cytokine (B) levels in neck tissue of saline + DNFB, abdominal tissue of nalbuphine + DNFB, and neck tissue of nalbuphine + DNFB compared to abdominal tissue of saline + DNFB.

A.

	Saline + DNFB Neck	Nalbuphine + DNFB Abdomen	Nalbuphine + DNFB Neck
CCL-1	1.53	1.24	4.34***
CCL-2	16.48****	1.77	59.79****
CCL-3	13.26****	2.20*	57.46****
CCL-4	1.97	1.52	1.52
CCL-5	1.88	1.42	3.92**
CCL-12	3.73**	1.45	10.75***
C5a	7.67***	1.45	13.46****
CXCL1	4.14**	1.12	9.01***
CXCL2	11.22***	1.87	82.84****
CXCL9	6.22***	1.43	13.14****
CXCL10	4.26**	1.03	6.76**
CXCL11	1.91	1.10	3.05*
CXCL12	1.68	1.01	2.03
CXCL13	1.26	1.03	0.98

B.

IL-1a	1.03	2.31*	1.74
IL-1ß	4.80**	1.67	15.71***
IL-1rd	2.60**	3.61**	4.35**
IL-2	1.20	1.19	1.54
IL-3	1.10	0.93	1.19
IL-4	1.27	0.97	1.50
IL-5	1.09	0.94	1.45
IL-6	1.48	0.99	1.96
IL-7	1.80	1.18	2.26*
IL-10	1.66	1.49	1.90
IL-12p7c	1.94	1.48	2.88*
IL-13	5.43**	2.88**	3.52**
IL-16	10.25***	5.80**	16.75****
IL-17	2.23*	1.48	3.54*
IL-23	2.42*	1.74	3.08*
IL-27	1.26	1.12	1.57
TIMP-1	6.56***	1.48	12.60****
G-CSF	1.55	1.05	2.19*

A.

	Saline + DNFB Neck	Nalbuphine + DNFB Abdomen	Nalbuphine + DNFB Neck
GM-CSF	1.46	1.10	2.39 *
M-CSF	3.93 **	1.79	10.03 ***
TNF-α	2.37 *	0.92	3.72 *
TREM-1	4.93 **	1.43	25.01 ****
SICAM	5.20 ***	3.40 **	4.70 ***
IFNγ	3.40 *	1.91	3.05 *

* P<0.05,

** P<0.01,

*** P<0.001,

**** P<0.0001)