

Investigating the effect of Ultra-low dose naloxone on spinal BDNF and KCC2 cotransporter in morphine tolerant and hyperalgesia rats

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Abstract

Background: Chronic opioids administration could lead to several side effects including morphine tolerance and opioid induced hyperalgesia (OIH). Tolerance and hyperalgesia to opiates reduce their effectiveness in the treatment of severe pain. Although the mechanisms are unclear. Recently, we have shown that repeated morphine treatments induced increases in spinal PKC α and GAT-1 expression. In this study we investigate the BDNF and KCC2 expression through ultra-low dose of naloxone coadministration of morphine.

Results: In morphine group, rats received 10 mg i.p. morphine and in treatment group 15ng ultra-low dose of naloxone with morphine for consecutive 8 days. Behavioral tests were performed on day 1 before and after the morphine injection, day 5, 8 (tolerance test) and 10, 48h after last morphine injection (opioid induced hyperalgesia OIH test). A number of rats were sacrificed on day 8 and others on day 10, then expression of BDNF and KCC2 were analyzed by western blot and immunohistochemistry techniques, respectively. Behavioral tests suggested that following 8 days of chronic morphine injection tolerance developed. OIH was shown 48 hours after the last morphine injection. Expression of BDNF significantly was increased and KCC2 downregulated in rats that developed morphine tolerance and OIH respectively. Ultra-low dose of naloxone by decreasing BDNF and increasing KCC2 was able to suppress development of OIH and alleviated morphine tolerance.

Conclusions: Our data suggest that BDNF and KCC2 maybe candidate molecules which are involved in tolerance and OIH. Ultra- low dose of naloxone along morphine might be a valuable therapeutic potential for controlling hypersensitivity following chronic morphine administration.

1. Background

It has been shown that long-term administration of opioids, including morphine, leads to tolerance, dependence, addiction, and hyperalgesia. Among the multiple adverse side effects, tolerance and opioid-induced hyperalgesia are importance. Although morphine remains the best analgesic of all the opioids (1), studies have shown that continuous injection of morphine can increase spinal BDNF release, which is involved in the development of hyperalgesia. This is due to the effect of BDNF, which binds to the tyrosine kinase receptor (TrkB) and then inhibits KCC2 cotransporter activity. KCC2 increases the accumulation of chlorine and potassium ions in the cell and produces sensitivity and excitability (2, 3). KCC2 levels downregulate by BDNF release following chronic morphine administration (3–5). Rivera (1999) presented several pieces of evidence showing that BDNF down-regulates KCC2, leading to impairment of Cl⁻ extrusion from neurons. Researchers have shown an obvious downregulation of KCC2 mRNA expression by BDNF (6). BDNF-induced hyperexcitability has been proposed to be partly due to effects on Gamma-aminobutyric acid (GABA) inhibition (7). The changes in intraneuronal Cl⁻ affect the amplitude and polarity of GABAergic responses (8) and are involved in the control of both neuronal and interstitial volume, which play a critical role in the modulation of neuronal excitability (9).

Opioid receptor antagonists, such as naloxone and naltrexone, at mg or μg . doses antagonize opioid functions, but at ng or pg. doses enhance the analgesic effect of opioid agonists (10). Co-administration of morphine with an ultra-low dose of naltrexone or naloxone potentiate the antinociceptive effect of morphine (11). Moreover, it has been shown that ultra-low-dose naloxone modulates spinal neuroinflammation and enhances the antinociceptive effect of morphine (12). These data suggest that ultra-low-dose naloxone might be clinically valuable in pain management (13) as co-infusion of ultra-low-dose naloxone along with morphine maintains the antinociceptive effect of morphine (12). Furthermore, the ultra-low-dose of naloxone is known as an effective adjuvant to suppress opioid tolerance and dependence (14). The mechanism underlying an ultra-low dose of naloxone is not well known. In this study, we examined the effect of co-administration of morphine and ultra-low-dose naloxone on BDNF and KCC2 expression in dorsal horn neurons in morphine-treated rats.

2. Results

2.1. Tail flick test on day 1, 5 and 8 after morphine injection

Four-hour tail-flick test results in the morphine group compared with the sham group showed the antinociceptive effect of morphine. Indeed, morphine created the maximum possible analgesic effects (MPAE). This effect remained significant in the sham group, although it decreased gradually from 120 min after injection to 240 min. Also, the results showed that co-administration of the ineffective dose of naloxone along with morphine the first day sustained the antinociceptive effect of morphine when compared to the sham group for up to three hours but decreased from 180 minutes to 240 min. However, it was not able to sustain the analgesic effect when compared to the morphine group (Figure 1).

On day 5, the four-hour tail-flick test in the morphine-treated group as compared with the sham group showed the antinociceptive effect of morphine within the first 60 minutes after injection. This effect gradually decreased from 60 min after injection to 120 min and continued decreasing until 240 min when it became similar to the sham group. The results of the same test showed that in the ineffective dose of co-administration of naloxone along with morphine up to day 5, the analgesic effect of morphine could be maintained for two hours, but after 120 minutes gradually decreased and became similar to the sham group (Figure 2).

On day 8, the results of the tail-flick test in the morphine-treated group as compared to the sham group showed an analgesic effect within the first 30 minutes after injection. This effect gradually decreased from 30 min to 240 min when it approached the sham group. Results of the same test showed in the ineffective dose of co-administration of naloxone along with morphine up to day 8 that the antinociceptive effect of morphine was maintained for up to 240 minutes and was significant when compared to the sham and morphine groups (Figure 3).

The results of an ineffective dose of naloxone for 8 days showed no significant antinociceptive effect on the experimental days.

2.2. Thermal hyperalgesia between days 1 and 10 using Hargreaves test

The results of the withdrawal plantar test, which measure thermal hyperalgesia, showed there was no significant difference between experimental groups on the first day before drug injection. Eight consecutive days of morphine injection showed thermal hyperalgesia on day 10 (48 hours after the last morphine injection) and before drug injection. A withdrawal plantar test on day 10 showed significant hyperalgesia in the morphine group, but in the ineffective dose of co-administration of naloxone along with morphine group there was no significant paw withdrawal when compared to the sham group. The naloxone groups did not show any significant antinociceptive effect (Figure 4).

2.3. BDNF protein expression changes following chronic administration of morphine

Western blot analysis showed the administration of morphine for 8 consecutive days increased BDNF protein expression in spinal dorsal horn neurons on days 8 and 10. Co-administration of an ineffective dose of naloxone along with morphine decreased the BDNF expression. The decreased expression of BDNF on day 8 was greater than day 10. There were no significant changes in BDNF expression between the ineffective dose of naloxone and sham groups (Figure 5).

2.4. KCC2 expression Following chronic administration of morphine using immunohistochemical technique

Immunohistochemical analysis of KCC2 in the lumbar section of the spinal cord following chronic administration of morphine for 8 consecutive days showed a significant decrease of KCC2 on day 8 (tolerance) and day 10 (hyperalgesia) when compared to the sham group. Co-administration of 10ng daily injection of naloxone along with morphine increased the expression of KCC2 in the spinal dorsal horn neurons on days 8 and 10 when compared to the sham group (Figure 6). Since both results on days 8 and 10 were similar and there were no significant changes of KCC2 in the dorsal horn neurons between days 8 and 10, we indicated day 10 images.

3. Discussion

Our study showed that intraperitoneal administration of morphine for 8 continuous days led to morphine antinociceptive tolerance and thermal hyperalgesia in rats. Coadministration of an ultra-low dose of naloxone along with morphine showed a controversial role in tolerance. In other words, on the first day that morphine showed maximum antinociceptive effect naloxone didn't have any effect, but gradually by the manifestation of tolerance and hyperalgesia, naloxone was able to improve and reduce tolerance and hyperalgesia by gradually manifesting them especially at day 8 and 10, respectively. It seems that naloxone had no effect until tolerance or hyperalgesia were established. Thus, our results showed the improving effects of coadministration of naloxone along with morphine but not alone. Our results were in agreement with previous studies. It has been previously demonstrated that chronic injection of

intraperitoneal, subcutaneous, or intrathecal morphine produce tolerance and hyperalgesia (15, 16). However, the development of hyperalgesia depends on the duration and dose of morphine (17). For example, King et al. (2005) showed that 6 days of SC. morphine administration via pellets created thermal hyperalgesia. In this regard, Hong et al. in 2010 and Powell et al. in 2000 showed a reduction in the analgesic effect of morphine and development of tolerance after 7 days of intrathecal continuous administration in animals (18, 19). In contrast, Gu et al. (2005) showed that 6 days of i.t. chronic morphine infusion (40 nmol/ μ L/h) would not develop hyperalgesia (20). In 2015 Zuo et al. observed mechanical and thermal hyperalgesia symptoms in animals after daily intraperitoneal injection of 10 mg/kg morphine (21). Different studies have indicated that the co-administration of opioid antagonists can greatly prevent the side effects of long-term opioids administration. New studies have shown that opioid receptor antagonists, such as naloxone and naltrexone, have anti-inflammatory effects at ultra-low doses and can reduce or reverse the adverse effects of repeated opioid use (22). In 1995 Crain showed that co-administration of a very low dose of naloxone along with morphine reduces tolerance and withdrawal syndrome (23). In contrast, Wang et al. (2005) reported that the analgesic effects of co-administration of morphine and naloxone was reduced after 7 days. Similar to our results, they showed that naloxone alone was ineffective. Many pre-clinical studies have shown that an ultra-low dose of naloxone or naltrexone enhances the analgesic effects of opioids (11, 14). In this study, we use an ultra-low dose of naloxone along with morphine and observed improving morphine antinociceptive effect.

Different mechanisms occur in neurons and glia that play an important role in this phenomenon. The molecular mechanisms underlying opioid tolerance and hyperalgesia is thought to overlap considerably with those involved in chronic pain signaling (24). Wang in 2005 reported that treatment with the same ultra-low dose of naloxone alone had no analgesic effect. Another finding of this study is that since the analgesic effects of opioids on receptors are inhibitory and chronic opioid administration switches G protein coupling from Gi/o to Gs, concomitant administration of an ultra-low dose of naloxone decrease Gs coupling and enhances Gi/o binding (14, 25). The antinociceptive effects of ultra-low doses of naloxone (ng or pg.) or naltrexone are mediated by various mechanisms such as inhibition of opioid receptor signaling pathways in DRG neurons, the release of enkephalins, and neuro-inflammatory events triggered by microglia. Also, this dose of naloxone specifically antagonizes the coupling of opioid receptors to Gs proteins, adenylyl cyclase/G β γ signaling, and the pathway of excitatory signals in the spinal cord, and in turn, increases the tendency to pair with Gi/o thereby maintaining and enhancing morphine analgesia (13, 23, 25, 26). In subsequent studies in 2008 and 2009, Wang et al. stated that naloxone may bind to A filaments and prevent G protein switching from inhibitory to excitatory transcriptional pathways (27–29).

The results of molecular studies in hyperalgesia and tolerant animals showed that administration of morphine for 8 consecutive days resulted in increased expression of BDNF in spinal dorsal horn neurons. We also observed that daily injection of 15 ng of naloxone plus morphine prevented the increase in BDNF expression, and injection of naloxone at a very low dose without morphine did not affect BDNF expression. Studies have shown that continuous injection of morphine can increase spinal BDNF release,

which is itself involved in the development of hyperalgesia. This is also due to the effect that BDNF exerts on neurons by binding to tyrosine kinase receptors (TrkB) so that by inhibiting KCC2 cotransporter activity, it increases the accumulation of chloride and potassium ions in the cell as well as sensitivity and excitability (2, 3). BDNF-induced hyperexcitability has been proposed to be partly due to the effects on GABAergic inhibition, Coull et al. (2005) showed that BDNF has decreased the paw withdrawal threshold and manifested hyperalgesia. In their in vivo study, they added BDNF to the dorsal horn neurons of the spinal cord and observed less negative potential than the control neurons, which could be indicating a shift from inhibitory to excitatory GABA responses (4). In our belief, BDNF may have different roles during morphine administration rather than singular. Therefore, it is necessary to clearly understand the function of BDNF in morphine tolerance and OIH.

In the second part of this study, we used immunohistochemistry to investigate the distribution of kCC2 cotransporter expression in the transverse section of the spinal cord.

In the morphine group, the expression of kCC2 cotransporter showed a significant decrement. We also observed increased kCC2 cotransporter activity in the dorsal horn neurons compared to the morphine group in rats that received morphine plus an ultra-low dose of naloxone. There was no significant change in kCC2 expression in the ultra-low dose of naloxone saline group. As Kakazu stated in 2000 and Hubner in 2001, the maintenance of hyperpolarizing inotropic responses is dependent on kCC2 (30, 31). Rivera (1999) confirmed that kCC2 is the major extruder ion that triggers hyperpolarization postsynaptic inhibition in the CNS (6). Therefore, any dysfunction of this cotransporter results in the accumulation of chloride ions inside the cell, due to the increase of chloride ions inside the cell, causes GABAergic receptors to shift from inhibitory to excitatory. All of these conditions can lead to hyperalgesia behavior in animals.

Rivera et al. (2002) showed that following the addition of BDNF to the culture medium, the expression intensity of KCC2 decreased, which subsequently affected neuronal chloride flow. They presented evidence that BDNF regulates KCC2 downregulation and disruption of chloride flow by acting on the TrkB receptor (7).

In a clinical trial on patients undergoing colorectal surgery in 2015, Xiao et al. showed that the combination of an ultra-low dose of naloxone (0.25 µg/kg/h) with remifentanyl decreased the adverse effects of acute morphine administration, tolerance, postoperative pain scores, bowel function recovery time, and length of hospital stay (32). Another clinical trial in 2019 on thoracotomy patients receiving bupivacaine and naloxone (100 ng) compared to bupivacaine alone showed significantly longer postoperative pain relief (33). In contrast, a study by Hosseini et al. in 2019 on renal colic patients did not report a significant analgesic effect between the group receiving naloxone (0.001 mg/kg IV.) plus morphine (0.1 mg/kg) with the group receiving only morphine (34). We suggest that the simultaneous administration of an ultra-low dose of naloxone affects the expression and secretion of BDNF and KCC2 regulation and consequently reduces the tolerance and hyperalgesia induced by chronic morphine administration.

Since opioid receptors mediate the inhibition of adenylyl cyclase, and an ultra-low dose of naloxone antagonize excitatory functions opioid receptors through G protein binding, this pathway is likely to be involved (35). Also, there is a direct relationship between BDNF increasing and KCC2 decreasing (7), since naloxone in ultra-low-dose has an anti-inflammatory effect (22, 36, 37). Therefore, it is hypothesized that this secondary drug can reduce BDNF secretion and subsequently prevent the downregulation of KCC2, thereby reducing the tolerance and hyperalgesia induced by long-term use of morphine. Equivalent inhibition of the Gs coupling of CREB phosphorylation by naloxone and its binding to pentapeptide filament A seems to be the mechanism of action of a very low dose of naloxone or naltrexone (27–29).

According to the mentioned studies and our results, there seems to be a strong relationship between the activity of BDNF expression and downregulation of KCC2 cotransporter during chronic morphine administration and neuroinflammation. Since the use of naloxone at very low doses along with morphine has an anti-inflammatory effect and can decrease microglial activity (7, 36), we hypothesize that a ULD of naloxone may affect BDNF, decreasing secretion and subsequently prevent KCC2 downregulation, and thereby prevent the tolerance and hyperalgesia induced by long-term use of morphine. Considering that BDNF increased in morphine treated rats, and the ability of ultra-low dose of naloxone along with morphine to block the rise in BDNF expression, it is clear that decreased BDNF expression plays an important role in modulating KCC2 activity. Increased BDNF expression, in turn, leads to more alterations in nerve cell membranes that could eventually lead to the development of OIH and tolerance. Although it seems that ULD of naloxone affects BDNF, decreasing secretion and subsequently prevent KCC2 downregulation and could improve the analgesic effect of morphine, it should be noted the effects of ultra-low doses of naloxone are mediated by various mechanisms.

4. Conclusion

Combined with previous study our data suggests that BDNF and KCC2 maybe candidate molecules that are involved in tolerance and OIH. By increasing KCC2, the ultra-low dose of naloxone along with morphine might be a valuable therapeutic potential for controlling hypersensitivity following chronic morphine administration.

5. Materials And Methods

5.1. Animal and experimental design

All experimental protocols were followed by guidelines on the ethical standard for investigation of morphine tolerance and hyperalgesia in animals by the Animal Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1397.272). Experiments were done on forty-eight adult male Wistar rats (Pasture Institute – Tehran, Iran), weight 180–220g that were housed three rats per cage and placed under a 12 h light/dark cycle in a room with controlled temperature (22 ± 1 °C) for ten days. Animals had ad libitum access to food and water.

Animals were allowed to habituate to the housing circumstances for several days before the experiments began. Behavioral studies were performed in a quiet condition. Efforts were made to use the minimum number of animals necessary to achieve statistical significance.

Rats were randomly separated into 8 groups with 6 rats in each group and divided to tolerance and OIH groups: i) sham, ii) morphine (10 mg), iii) treatment group: morphine 10 mg and naloxone (NAL, 15 ng), and iv) naloxone (15 ng) and saline (SAL) Table 1.

In the sham group, rats received saline, in the morphine group, rats received (IP) 10 mg morphine i.p. in the treatment group, rats received 15ng ULD of naloxone i.p., along with morphine, and in the saline group, rats received ULD of naloxone along with saline for 8 consecutive days. Behavioral tests were performed to evaluate tolerance on day 1 before and after the morphine injection, and days 5 and 8 by the tail-flick test (TL) (Borje Sanat Co. Iran); and on day 1 before morphine injection and day 10, 48h after last morphine injection to evaluate OIH by plantar test (Ugo Basile, Italy). Three rats from all groups were euthanized under (2.0-2.5%) Isoflurane inhalation anesthesia on day 8 and the Lumbar segments of the spinal cord were collected (to study BDNF expression by the western blotting method). On day 10 three rats from all groups were deeply anesthetized by ketamine (10%) and xylazine (2%) i.p. and transcardially perfused with phosphate-buffered saline. Spinal cords were isolated and lumbar segments were removed (to study KCC2 expression by the immunohistochemistry method). All experiments were done from 8 am until 4 pm.

Table 1. Characteristics of experimental groups

Group	n	Treatment for 8 days
Sham (tolerant) Sham (OIH)	12	Saline
Morphine (tolerant) Morphine (OIH)	12	Morphine 10mg/kg daily i.p.
Morphine+ Naloxone (tolerant) Morphine+ Naloxone (OIH)	12	Morphine 10mg/kg+Naloxone 15ng daily i.p.
Naloxone+ Saline (tolerant) Naloxone+ Saline (OIH)	12	naloxone 15ng +saline daily i.p.

5.2. Instruments and Reagents

Hargreaves Apparatus (Ugo Basile, Italy), tail-flick (Borje Sanat, Iran), centrifuge, polyvinylidene difluoride (PVDF) membrane, Bio-Rad immunoblotting apparatus ECL chemiluminescence, optical Olympus AX70 microscope (Japan) with a DP11 digital camera, Morphine sulphate (Temad Co, Iran), naloxone hydrochloride (Santa Cruz, USA), Isoflurane (Primal Critical Care, UK), ketamine (Sigma, USA), xylazine (Sigma, USA).

5.3. Behavioral tests

The plantar test (Hargreaves method) was used to assess the development of thermal hyperalgesia. Rats were placed in the plexiglass chambers to adapt to the lab environment 30 minutes before the initiation of tests. A light beam, as the heat source, was focused on the middle surface of the rat's right hind paw. The time interval between the initiation of the light beam and the rapid withdrawal of the hind paw was considered as the nociceptive threshold. A cut off time of 30 seconds was set to prevent tissue damage. The plantar test was performed on day 1, before the first injection of morphine and on day 10, 48 hours after the last morphine injection. On each day, 5 trials were made with 5 minutes interval and mean paw withdrawal latency (PWL) was obtained. A significant decrease in PWL from baseline in day 1 was defined as thermal hyperalgesia.

The development of morphine analgesic tolerance was examined using the tail-flick test. Rats were placed on the tail-flick apparatus before the injection of Morphine on days 1, 5, and 8. Radiant heat was administered to the caudal end of the tail. A cut off time of 20 seconds was established to minimize tissue damage. In each session, 3 trials were conducted with 3 minutes in between each trial. The mean tail-flick latencies (TL) of the 3 trials was set as the baseline latency (BL).

For acquiring absolute tail-flick latency and percentage of maximum possible analgesic effect (%MPAE) ($\%MPAE = [(TL - BL) / (20 - BL)]$) of morphine, a single measurement of tail-flick latency was made at 30, 60, 120, 180 and 240 minutes after the injection of morphine.

5.4. Western blotting

On days 8 and 10, three rats from all groups were sacrificed. Western blotting was performed on lumbar segments of spinal cord homogenates to determine BDNF protein expression levels. The whole lumbar segment of the spinal cord was homogenized in lysis buffer, and the total protein extract was obtained by centrifugation at 13,000g for 40 min at 4 °C. Protein concentration was determined by Bradford assay, and equivalent protein amounts of each sample were separated using dodecyl sulfate polyacrylamide gel electrophoresis sodium dodecyl sulfate page (SDS-PAGE). The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes using a Bio-Rad immunoblotting apparatus. Afterward, to block non-specific binding sites, membranes were incubated in blocking buffer bovine serum albumin (BSA 5%) for 80 minutes. Membranes were then incubated overnight to block non-specific binding sites; membranes were incubated in blocking buffer (BSA 5%) for 80 minutes. Membranes were then incubated overnight with the BDNF antibody (AB203573, 1:5000). The next day, blots were washed three times with 0.1% Tris Buffered Saline Tween (TBST) 20 and Tris Buffered Saline (TBS) each for 10 minutes, and then incubated with horseradish peroxidase-conjugated secondary antibody in blocking buffer (SC-516102, 1:10000) for 85 minutes. After washing the blots three times with TBST, protein complexes were visualized using ECL chemiluminescence (Amesrhram). After scanning the X-ray films, the protein expression was quantified and analyzed by ImageJ 1.44 NIH software. Data were represented as the ratio of the density of BDNF bands to β -actin bands.

5.5. Immunohistochemistry analysis

The expression of KCC2 in the lumbar segments of the spinal cord was assessed by immunohistochemistry analysis. On day 10 three rats from all groups were deeply anesthetized by ketamine (10%) and xylazine (2%) and trans cordially perfused with phosphate-buffered saline (PBS), followed by freshly prepared 4% phosphate-buffered paraformaldehyde (pH 7.4). Spinal cords were isolated and lumbar segments transected after cardiac perfusion with 4% paraformaldehyde. Next, the lumbar segments of the spinal cords were removed and post-fixed in 4% paraformaldehyde for 48 hours. Lateral sections with a 5 μ m thickness from lumbar segments of the spinal cords were prepared using a microtome rotatory apparatus. Immunohistochemical analysis of KCC2 was performed on paraffin-embedded sections. First, sections were placed in the oven for 20 minutes, then deparaffinized in xylene and rehydrated. Next, sections were pacified in 3% hydrogen peroxide (H₂O₂) and then exposed to heat mediated antigen retrieval using citrate buffer (pH 6) for 50 minutes. Subsequently, sections were blocked to prevent non-specific binding, prior to incubating them overnight with primary antibody (MAB=16982, 1/300) at 4 °C. The sections were then washed with wash buffer and incubated with a ready-to-use biotinylated secondary antibody (PAB0096), followed by the addition of a streptavidin-conjugated enzyme. After immunoreactions, sections were washed with wash buffer and then color development performed using 3,3'-diaminobenzidine (DAB) solution for 10 minutes. Slides were then counterstained with hematoxylin and dehydrated using graded alcohols and xylene. Finally, sections were mounted onto slides, following coverslip application. The images were taken using an optical Olympus AX70 microscope (Japan) with a DP11 digital camera. The immunoreaction intensity was analyzed with NIH Image J software. The values were obtained by the densitometric analysis performed by ImageJ software. The background intensity was subtracted from the intensity in the areas of interest. Average intensities were obtained from 3 animals in each group.

6. Statistical Analysis

In this study, data were expressed as mean \pm standard (S.E.M.). GraphPad Prism (version 6.0; GraphPad Prism Software Inc., San Diego, CA, USA) was used for statistical analysis. Data from behavioral tests were examined between groups by two-way ANOVA analysis followed by Tukey's post-hoc test for multiple comparisons. Western Blot analysis were compared by one-way ANOVA followed by Tukey's post-hoc test. Quantitative analysis of immunohistochemical images was performed by t-test. P-values less than 0.05 were considered as statistically significant.

Abbreviations

BDNF: Brain-Derived Neurotrophic Factor; BL: Baseline Latency; BSA: Bovine Serum Albumin

CREB: cAMP (Adenosine 3'5' cyclic monophosphate) Response Element Binding Protein; DAB: Diaminobenzidine; GABA: Gamma-aminobutyric acid; GAT-1: GABA transporter 1; Gi: G protein inhibiting; Gs: G protein stimulating; IV: Intravenous; KCC2: K⁺, CL⁻ Cotransporter 2; mg: Milligram; MOR: Morphine; MPAE: Maximum possible analgesic effect; NAL: Naloxone; Ng: Nanogram; OIH: Opioid-Induced Hyperalgesia; PBS: Phosphate buffered saline; pg: Picogram; PKC α : Protein Kinase C α ; PKC β : Protein

Kinase C β ; PVDF: Polyvinylidene difluoride; PWL: Paw Withdrawal; SAL: Saline; SC: Subcutaneous; SDS-PAGE: Sodium dodecyl sulfate page; TBST: Tris Buffered Saline Tween-20; TL: Tail flick Latency; TrkB: Tyrosine Kinase B; ULD: Ultra-Low Dose;

Declarations

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Ethics approval and consent to participate

All experimental protocols were followed by guidelines on the ethical standard for investigation of morphine tolerance and hyperalgesia in animals by the Animal Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1397.272).

Consent for publication

A copy of the consent form is available for review by the Editor of this journal.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interests.

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Authors' contributions

Homa planned, designed and conducted experiments. Mozghan did all the experimental works. Jalal and Samira contributed some part of western blotting and of immunohistochemistry analysis interpretation respectively and revised some aspect of manuscript. All authors have read and approved the final manuscript.

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Figures

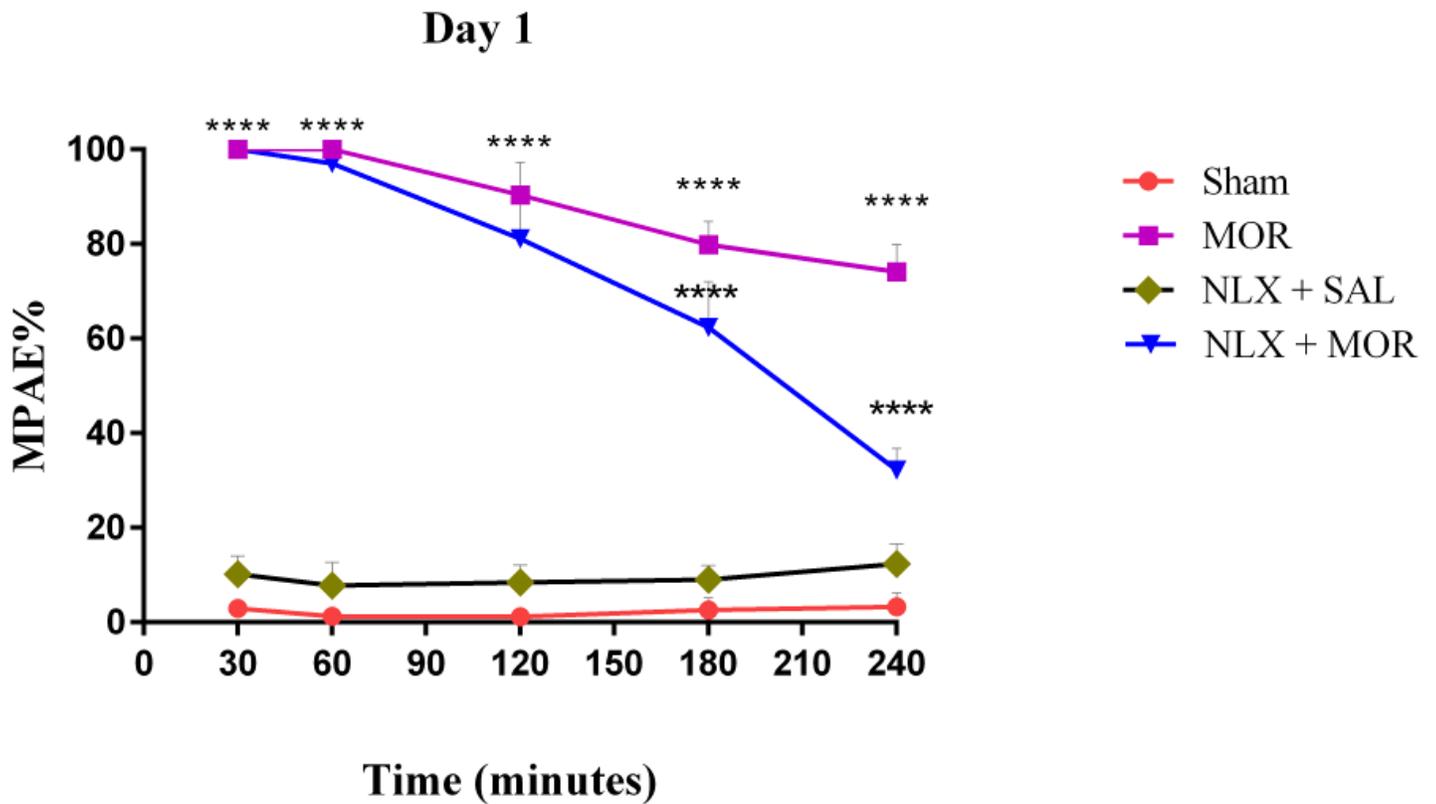


Figure 1

Maximum antinociceptive effect (MPAE%) after morphine injection on the first day. Tail flick test was measured at 30, 60, 120, 180 and 240 minutes after drug injection. In the MOR group, the antinociceptive effect of morphine decreased gradually from 120 min after injection to 240 min but remained significant when compared to the sham group. An ineffective dose of naloxone did not produce the antinociceptive effect in comparison to the sham group. NLX + MOR was able to produce a significant antinociceptive affect up to 180 minutes after injection when compared to the sham but gradually decreased after three hours. However, it was not able to sustain the analgesic effect when compared to the morphine group. The analgesic effect has shown as a percentage. P <0.05 was considered a significant difference. (P <0.01 **, *** P <0.001, **** P <0.0001)

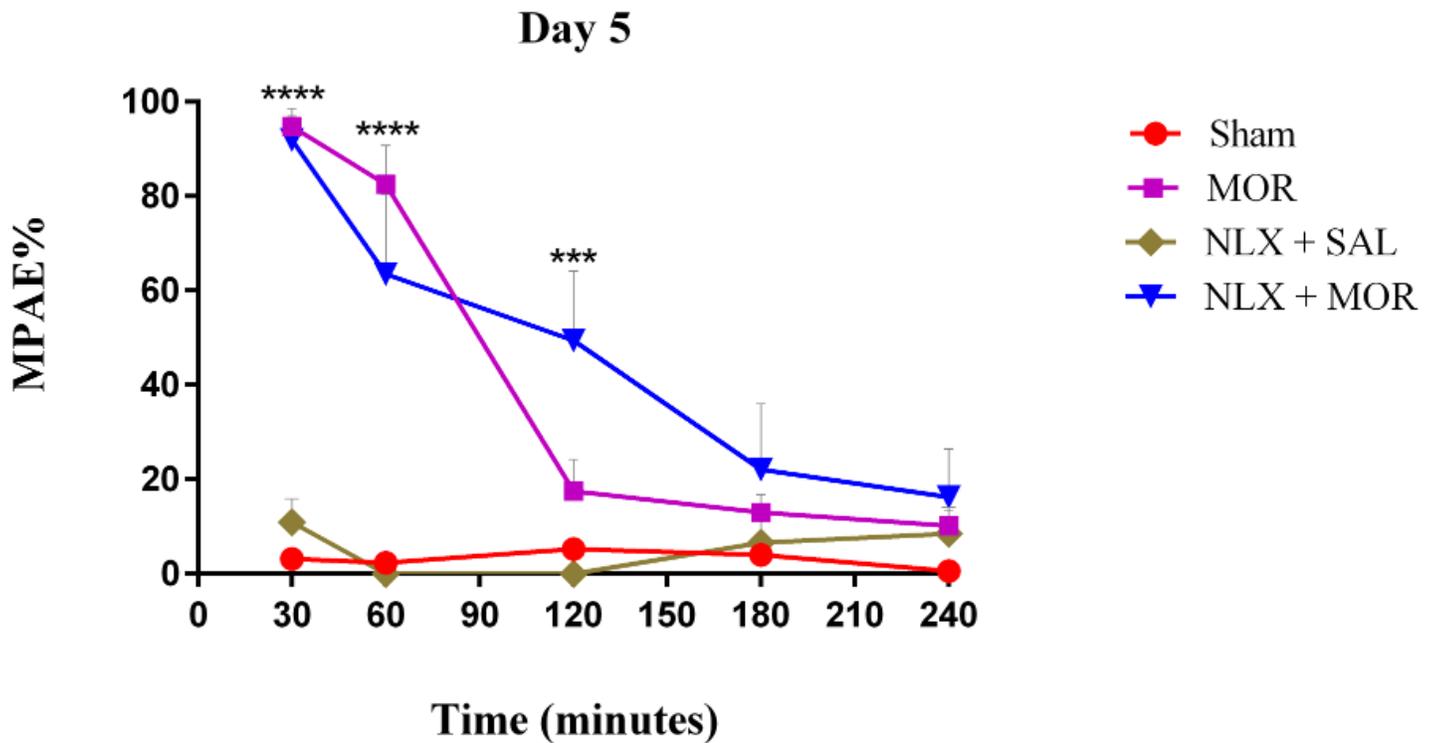


Figure 2

Maximum antinociceptive effect (MPAE%) after morphine injection on day 5. Tail flick test was measured at 30, 60, 120, 180 and 240 minutes after drug injection. The four-hour tail-flick test in the morphine-treated group compared with the sham showed the maximum antinociceptive effect of morphine within the first 60 minutes after injection. It was gradually decreased from 60 min after injection to 120 min and continued until 240 min and became similar to the sham group. The results of the same test showed that in the ineffective dose of co-administration of naloxone along with morphine up to day 5, the antinociceptive effect of morphine could maintain for two hours, but after 120 minutes gradually decreased and became similar to the sham group. The effect of analgesia has shown as a percentage. $P < 0.05$ was considered a significant difference. ($P < 0.01$ **, *** $P < 0.001$, **** $P < 0.0001$)

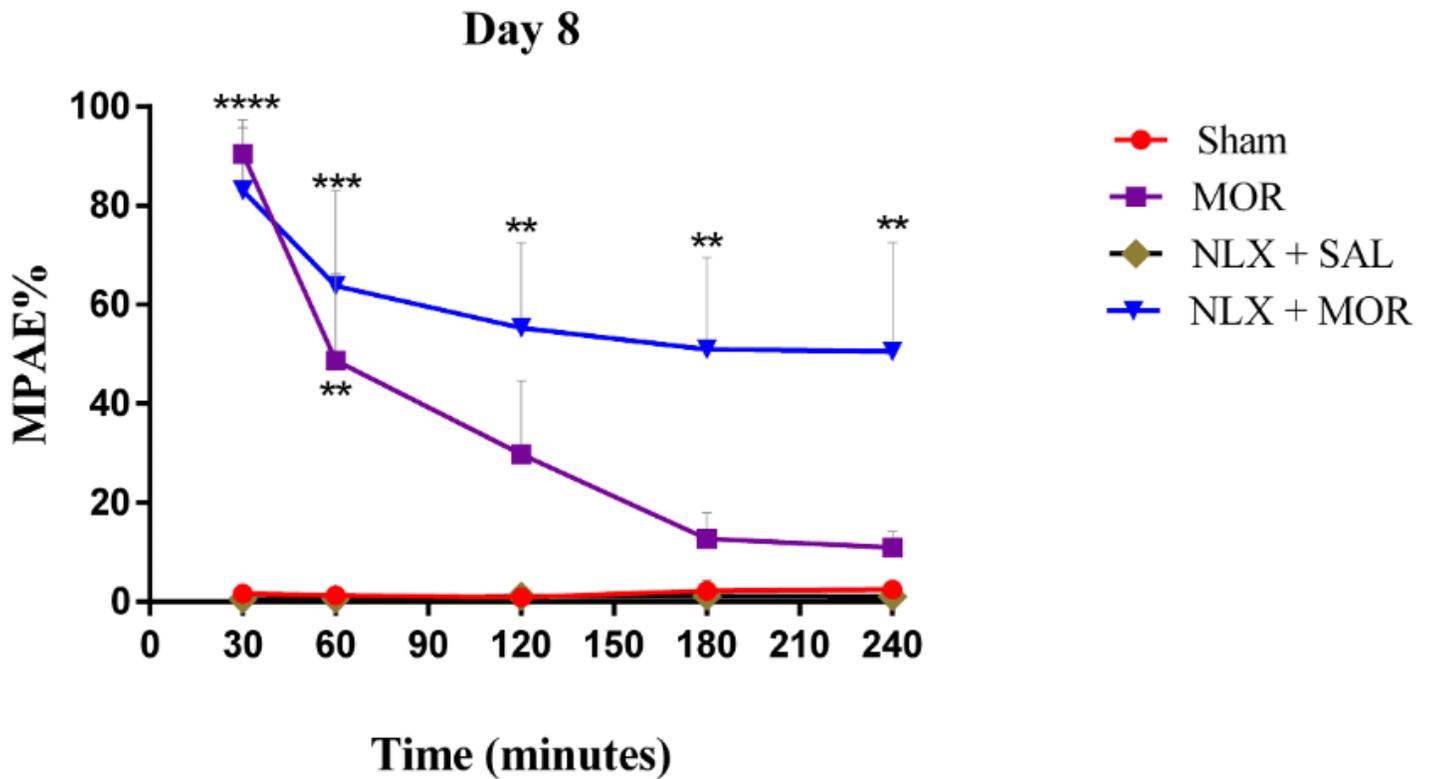


Figure 3

Maximum antinociceptive effect (MPAE%) after morphine injection on day 8. Tail flick test was measured at 30, 60, 120, 180 and 240 minutes after drug injection. The four-hour tail-flick test on day 8 in the MOR group compared with the sham group showed an antinociceptive effect of morphine within the first 30 minutes after injection. This effect gradually decreased from 30 to 240 minutes and became similar to the sham group. The results of the same test showed that in the ineffective dose of co-administration of naloxone along with morphine up to day 8 the antinociceptive effect of morphine maintained up to 240 minutes and was significant when compared to the sham and morphine groups. The numbers are shown as the maximum analgesic effect. $P < 0.05$ was considered a significant difference. ($P < 0.05$ *, *** $P < 0.001$, **** $P < 0.0001$).

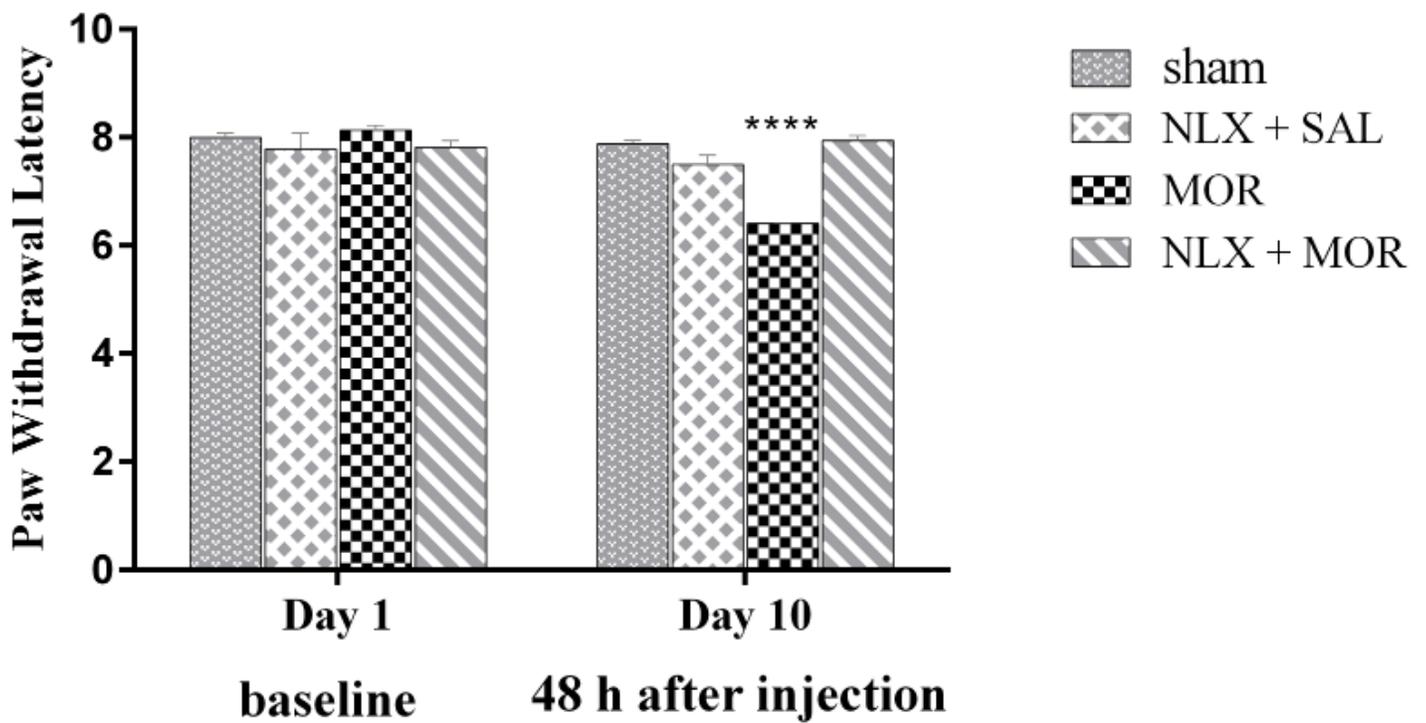


Figure 4

The results of the withdrawal plantar test which measure thermal hyperalgesia showed that there was no significant difference between experimental groups on the first day before drug injection. Consecutive 8 days of morphine injection showed thermal hyperalgesia on day 10 (48 hours after the last morphine injection) and before drug injection. Withdrawal plantar test on day 10 showed significant hyperalgesia in the morphine group but in the ineffective dose of co-administration of naloxone along with morphine group, there was no significant paw withdrawal when compared to sham. The naloxone groups did not show any significant antinociceptive effect. $P < 0.05$ was considered as the least significant difference. $P < 0.0001$ **** = Comparison of thermal hyperalgesia between morphine and sham groups

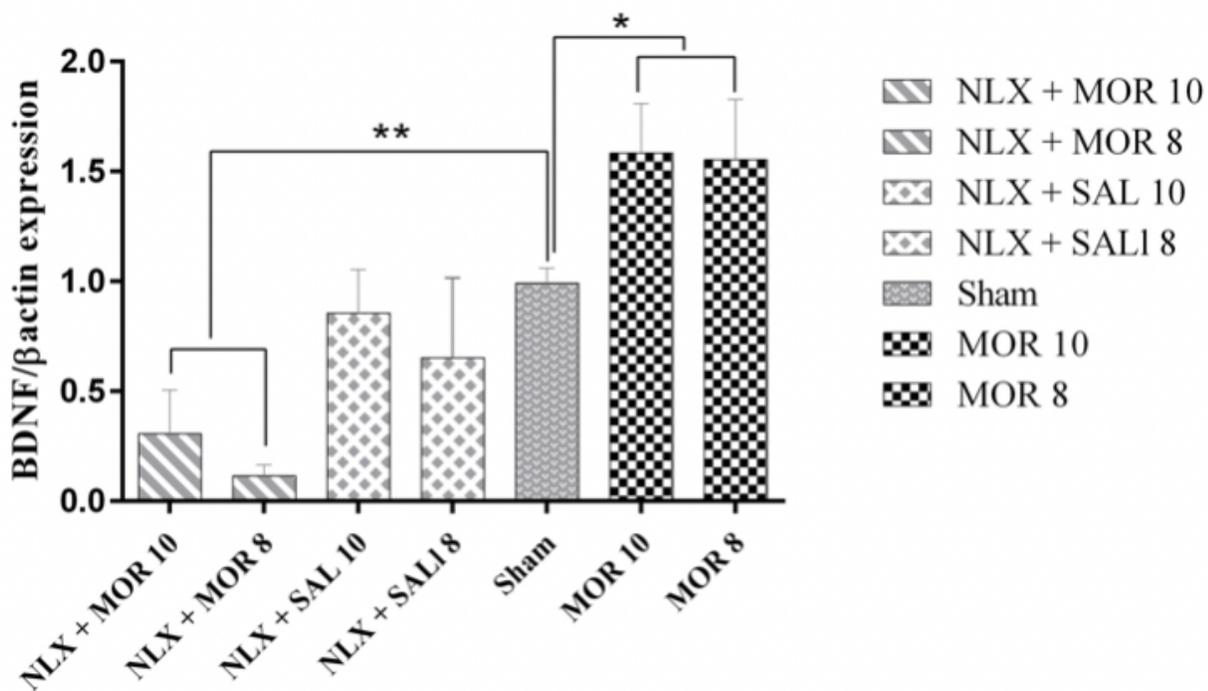
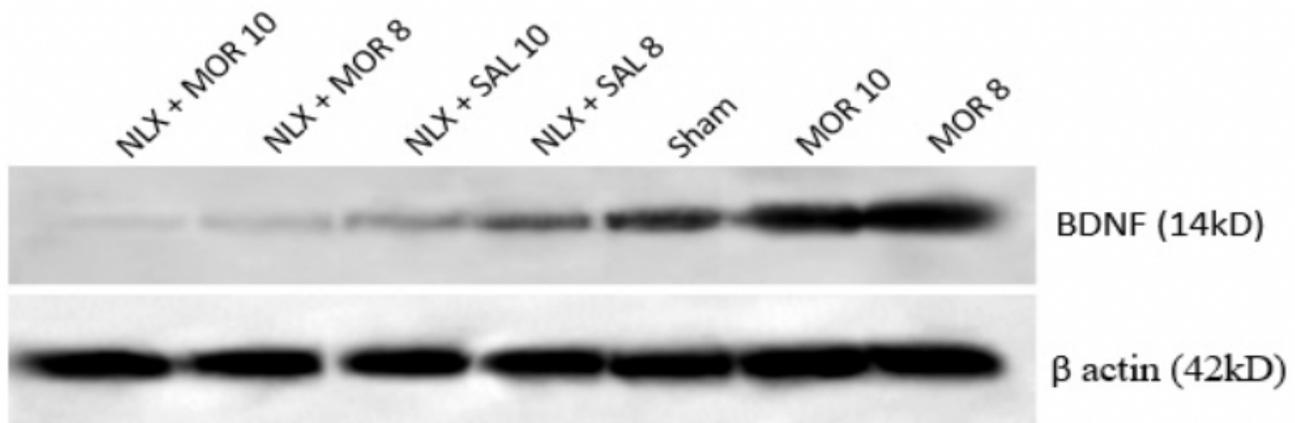


Figure 5

The chart of altered expression of BDNF and β actin proteins following chronic administration of morphine. Chronic administration of morphine for consecutive 8 days increased the expression of BDNF in the spinal dorsal horn neurons on days 8 and 10. BDNF expression in the NLX + SAL group was similar to the sham group. Co-administration 10ng daily injection of naloxone along with morphine decreased the BDNF expression on days 8 and 10. The decreased expression of BDNF on day 8 was greater than day 10. Results were compared by BDNF bands to β actin. (****P <0.0001).



Figure 6

Immunohistochemical analysis of KCC2 in lumbar section of spinal cord following chronic administration of morphine for consecutive 8 days showed a significant decrease of KCC2 on day 8 (tolerance) and 10 (hyperalgesia) when compared to the sham group. Co-administration 10ng daily injection of naloxone along with morphine increased the expression of KCC2 in the spinal dorsal horn neurons on days 8 and 10 when compared to sham group. Since both results on days, 8 and 10 were similar and there were no significant changes of KCC2 in the dorsal horn neurons between days 8 and 10 we indicated day 10 images. Microscopic images of the intensity of KCC2 cotransporter in the spinal cord in Sham, MOR, NLX + MOR and NLX + SAL groups are shown with two magnifications 10* and 40*. (*P <0.05, ** P <0.01).

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