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ML171, a specific inhibitor of NOX1 attenuates formalin induced nociceptive sensitization by inhibition of ROS mediated ERK1/2 signaling

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ABSTRACT

Reactive oxygen species (ROS) have a key role in different etiologies of pain. At sub-cellular level, mitochondria and plasma membranes have been identified as endogenous sources of ROS required for pain generation. NADPH oxidase (NOX) is the main contributor of membrane associated ROS generation. Out of 7 isozymes, NOX1, NOX2 and NOX4 are reported to be associated with nociceptive sensitization. Therefore, it has been hypothesized that specific inhibition of the NOX isozymes could be putative strategy for treatment of pain. However, unavailability of specific inhibitors was the biggest obstacle to test this hypothesis. Here, we investigated anti-nociceptive potential of a newly identified specific NOX1 inhibitor ML171 in formalin induced inflammatory pain. ML171 administration decreased the paw lickings and flinching response in both phases of formalin test. Behavioral response was supported with decreased activation of c-Fos in spinal dorsal horn. The increased level of total NOX activity, ROS and pERK1/2 in dorsal root ganglion (DRG) and spinal dorsal horn of formalin induced nociception were reversed by ML171 administration. ML171 also inhibited the upregulated Tumor necrosis factor receptor 1 (TNFR1) expression in DRG, whereas did not show any effect in spinal dorsal horn which was unaltered after formalin insult. The study for the first time depicts anti-nociceptive potential of ML171 via regulation of ROS mediated ERK1/2 signaling by inhibition of NOX1 activity.

1. Introduction

ROS including superoxide, peroxynitrite, hydroxyl radical, and hydrogen peroxide, are critically involved in different etiologies of pain (Borin-Yamacita et al., 2015; Cameron et al., 2001; Little et al., 2013; Singh and Vinayak, 2017a). Pharmacological removal of these ROS by nonspecific ROS scavengers and antioxidants effectively prevent and reverse development of nociception (Singh and Vinayak, 2017b; Singh and Vinayak, 2015). ROS is produced by various cellular enzymes showing multiple sources as byproducts of aerobic respiration or produced purposefully by neutrophil against injury/infection. Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) is the major enzyme for superoxide generation (Doyle et al., 2010). NADPH oxidase (NOX) is membrane-associated, multiunit enzyme that produces superoxide using NADPH as an electron donor. Deregulation of NOX may have deleterious effects as NOX mediated ROS production plays critical role in cell physiology (Brown and Griendling, 2009). Among several isozymes of NOX identified so far, NOX2 and NOX4 has been implicated in neuropathic pain (Geis et al., 2017; Gerhardt et al., 2014). Although involvement of specific NOX isozyme in inflammatory pain is not established yet, NOX1 may be a plausible mediator of development and maintenance of inflammatory pain. Ibi et al. have shown novel role for superoxide-generating enzyme NOX1 in the development of inflammatory pain by genetic ablation studies (Ibi et al., 2008). Recently the implication of NOX1 has been shown in tissue repair by regulating inflammation (Fu et al., 2014). However, pharmacological inhibition of NOX1 is required to ascertain its role by providing deeper mechanistic insight, and would be more important for therapeutic utility. Many of the available NOX inhibitors lack isoform specificity and the mechanism of action is poorly understood (Gerhardt et al., 2013). Recently identified ML171 (2-acetylphenothiazine) is a specific inhibitor of NOX1, with only marginal activity on other ROS producing enzymes and receptors (Gianni et al., 2011). Based on the literature, anti-nociceptive potential of the specific inhibitor of NOX1, i.e. ML171 has been hypothesized. To validate the hypothesis, ML171 is selected to analyze its effect on nociceptive behavior and the molecular mechanism implicated therein.

Tumor necrosis factor-alpha (TNF-α) is the first cytokine triggered in response to oxidative stress, leading to release of other cytokines to sensitize nociceptors. TNF-α/TNFR1 signaling plays important role in

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central sensitization of spontaneous pain triggered by superoxide anion (Borin-Yamacita et al., 2015). The ERK1/2 (extracellular signal-regulated kinase) MAPK (mitogen-activated protein kinases) signaling pathway is crucial for initiation and maintenance of nociception. ERK1/2 is activated within few minutes after nociceptive stimulation. Most of the signaling pathways converse to ERK1/2 during development of nociception (Ji et al., 2008).

Therefore, the present study is designed to test the anti-nociceptive potential of NOX1 specific inhibitor ML171 in formalin induced pain by investigating its effect on nociceptive behavior as well as molecular parameters involved in ROS mediated ERK1/2 activation.

2. Materials and methods

2.1. Drugs and reagents

General chemicals were purchased from Sigma-Aldrich (Saint Louis, USA). ML171 was purchased from Tocris Bioscience (Avonmouth, Bristal, UK). Nicotinamide Adenine Dinucleotide Phosphate Reduced (NADPH) Tetrasodium Salt from Sisco Reasearch Laboratories Pvt. Ltd. (Mumbai, India), XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) from MP Biomedicals (CA, USA), Polyclonal anti-c-Fos rabbit antibody was purchased from Abcam (Cambridge, UK), Polyclonal anti-TNFR1 and anti-ERK1/2 antibodies from BioVision (Milpitas, CA, USA), monoclonal anti-pERK1/2mouse antibody from Cell Signaling Technology, Inc. (Danvers, Massachusetts, USA), and monoclonal anti-β-actin antibody from Sigma-Aldrich (Saint Louis, USA).

2.2. Animals and treatments

Swiss albino adult male mice of 12–14 weeks weighing approx. 25-30 gms were used for the experiments. All animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. All experiments were performed with the approval of Committee for the Purpose of Control and Supervision of Experiments on Animals (Licence:1802/GO/Re/S/15/CPCSEA). Mice were bred and maintained under standard laboratory conditions; at 22 ± 2 °C with 12 h light/dark schedule with *ad libitum* supply of standard animal feed and drinking water.

2.2.1. For behavioral analysis

Normal adult male mice (12–14 weeks) were distributed in five groups. 10 μl normal saline (0.9% NaCl) was injected (s.c.) to animals of first group (S) on dorsal side of right hind paw. The animals of other four groups received 10 μl of 5% formalin solution prepared in normal saline (s.c.) into dorsal side of right hind paw. Further group II (FD), III (FM10), IV (FM20) and V (FM30) received 50 μl dimethyl sulfoxide (DMSO), ML171 dose of 10 mg, 20 mg and 30 mg/kg body weight in 50 μl DMSO respectively via intra-peritoneal (i.p.) route. ML171 was injected 30min prier to formalin insult. The time interval of 30min prior to formalin insult was selected after behavioral studies on three different time points i.e. immediately (zero), 30min and 1hr before the formalin injection. The behavioral test in terms of flinch count and paw licking was started immediately after formalin injection into the hind paw for 90 min at an interval of 5 min.

2.2.2. For molecular and biochemical studies

Normal adult male mice (12–14 weeks) were distributed in four groups. Needle was pricked on dorsal surface of right hind paw of animals in group I (sham). Group II received 10 μl saline (saline), group III (FD) and group IV (FM20) received 10 μl of 5% formalin in saline. Further, animals of groups FD and FM received i.p. injection of 50 μl DMSO, and ML171 dissolved in 50 μl DMSO respectively 30min prior to formalin injection. Based on the behavioral data obtained, ML171 dose of 20 mg/kg body weight was selected.

2.3. Flinching and licking tests

Behavioral experiments were performed (as described by Tjolsen et al., 1992) in three sets of animals with pre-treatment of ML171 as (1) zero time (2) 30 min and (3) 1hr before formalin injection to determine effective pre-treatment time. Nociceptive response was observed after formalin injection. The saline or formalin injected animals were immediately placed in a transparent observation box. Nociceptive behavior was manually quantified by counting number of paw flinches and paw lickings at 5 min interval up to 90min. Total area under curve for flinch count for the two phases i.e. phase1 from time 0–15min (AUC_{0-15}) and phase2 from 25 to 90min (AUC_{25-90}) was quantified. Number of licks was represented as cumulative lickings. The observer was blinded to treatments given in different groups.

2.4. Immuno-histochemical staining of c-Fos

Animals were perfused and tissue was collected after 40min of formalin injection and processed as described earlier (Singh and Vinayak, 2016). Immuno-histochemical staining of c-Fos was performed in spinal cord sections (L3-L5) using standard protocol (Singh and Vinayak, 2016). Level L3-L5 of spinal cord was demarcated carefully as described by Harrison et al. (2013). c-Fos expression was observed in laminae I,II,III and IV cumulatively in ipsilateral dorsal horn. Rabbit anti c-Fos antibody (1:200) and horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibodies (1:500) were used. c-Fos positive cells were detected by DAB (di-aminobenzidine) staining. Stained sections were observed under a light microscope (Leitz "laburlux S" microscope, Earnst Leitz GmbH, Wetzlar, Germany) and images were taken with Leica DCF290 camera (Leica Microsystems Ltd., Germany). c-Fos positive cells were counted manually in 4–6 sections for each group and average was taken. A representative photograph is shown in the results.

2.5. Determination of ROS level

ROS level was measured by fluorometric method using 2′,7′-dichlorofluorescein diacetate (H2DCFDA) as previously reported (Das and Vinayak, 2014). DRG or spinal cord extracts containing equal amount of protein were incubated with equal volume of 50 μM H2DCFDA (Invitrogen) at 37 °C for 60 min. Fluorescence was recorded at 485 nm (excitation) and 530 nm (emission) with Biotek Synergy H1 Hybrid Multi-Mode Reader and presented in arbitrary units (AU) in terms of fluorescence intensity/mg protein.

2.6. Preparation of membranous fraction

Tissue homogenate was prepared in a lysis buffer containing 50 mM potassium phosphate buffer (pH 7.4), 5 mM EDTA, 2 mM EGTA, 0.5 mM PMSF and protease inhibitor cocktail and cell debris was removed. The supernatant was centrifuged at 100,000 g for 1 h at 4 °C in an ultracentrifuge (HITACHI micro ultracentrifuge CS 150NX). The cytosolic fraction after 100,000 g centrifugation was removed and pellet was resuspended and centrifuged at 15,000 g for 30 min at 4 °C; and resulting supernatant was collected as membrane rich fraction.

2.7. Assessment of NADPH oxidase (NOX) activity

Activity of NADPH oxidase in terms of superoxide production was assessed in membranous fraction of tissue homogenates of DRG and spinal cord according to the method described by Sutherland and Learmonth (1997), with minor modifications. Briefly, reaction mixture (1 ml final volume) containing sample in lysis buffer having 50 mM potassium phosphate buffer (pH 7.4), 5 mM EDTA, 2 mM EGTA, 0.5 mM PMSF and protease inhibitor cocktail; and 0.5 mM XTT, was prepared fresh. Reaction was started by adding 0.1 mM NADPH to the above

suspension and A_{470} was recorded for 10 min at an interval of 1min.

2.8. Western blotting

Dorsal root ganglia as well as spinal cord (L3–L5) were homogenized in 50 mM Tris-Cl (pH 7.6) containing150mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1% SDS, 0.5% sodium deoxycholate, 0.1% Triton X-100, 1 mM PMSF and protease inhibitor cocktail. The supernatant was used for Western blot analysis of pERK1/2, ERK1/2 and TNFR1. Equal amount of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with non-fat milk for 2 h to prevent non specific binding. Blots were incubated overnight with anti pERK1/2 (1:1000), anti ERK1/2 (1:1500), anti TNFR1 (1:1200) antibody; washed in PBS for 5–10min and incubated with HRP conjugated secondary antibodies (1:2500) for 2 h. Bands were detected by enhanced chemiluminescence (ECL) on X-ray film. Alpha Imager 2200 software (Alpha Innotech) was used for densitometric analysis. β-actin served as a loading control.

2.9. Statistical analysis

Flinch test and NOX1 activity was analyzed by repeated measure ANOVA followed by Tukey post hoc test. Rest of the results was analyzed by one-way ANOVA followed by Tukey post hoc test using Statistical Package for the Social Sciences (SPSS) software, IBM SPSS Statistics, IBM Corporation, New York, USA. Values were expressed as mean ± S.E.M. obtained from three different sets of experiments; $p < 0.05$ was taken as statistically significant.

3. Results

3.1. ML171 inhibits nociceptive behavioral responses after formalin injection

Subcutaneous injection of formalin produces an acute inflammatory nociceptive response. In the present study, the nociceptive behavioral response was checked in terms of number of flinches at 5 min intervals for 90 min following formalin administration. Formalin-induced mice displayed discrete biphasic behavioral response consisting of an early short lasting acute phase (0–15min) followed by a prolonged tonic phase (25–90min). Flinching response was peaked in tonic phase at around 35–40 min after formalin injection. The observation was further validated by analyzing area under curve i.e. $AUC_{0\text{-}15}$ representing phase1 and $AUC_{25\text{-}90}$ as phase2 (Fig. 1).

As there is no report of application of NOX1inhibitor ML171 on pain model, the dose was standardized taking three doses i.e.10, 20 and 30 mg/kg body weight. The time of ML171 administration was standardized as formalin leads to immediate nociceptive response. The three time points selected were (i) immediately (zero time), (ii) 30min, and (iii) 1hr before formalin injection. The difference in pre-treatment time affected flinching behavior in phase1. At zero time, ML171 administration caused no significant difference in flinching behavior with all the three doses i.e.10, 20 and 30 mg/kg body weight (Fig. 1A-i, ii). Pre-treatment time of 30min of formalin insult significantly attenuated flinch counts ($p < 0.05$) with 20 mg/kg body weight dose only (Fig. 1B-i, ii), and 60 min pre-treatment caused similar attenuation effect with 20 mg and 30 mg (Fig. 1C-i, ii). However in case of phase2, all the three doses, at all the three pretreatment times resulted in significant decrease ($p < 0.005$) in the flinch count as compared to DMSO treated group (Fig.A-i, iii; Fig. 1B-i, iii; Fig. 1C-i, iii).

The nociceptive response was further checked with paw licking behavior. Cumulative paw licking inclusive of both phases exhibited similar trend of response as observed in flinch behavior (Fig. 2). The effect of ML171 at all the three pre-treatment time points was dose dependent. The dose of 10 mg/kg body weight was found to produce insignificant effect as compared to DMSO treated group, however 20 mg/kg and 30 mg/kg body weight were able to attenuate licking behavior significantly ($p < 0.005$) at all the three pre-treatment time points. There was no significant difference in response with the two doses i.e. 20 mg/kg and 30 mg/kg body weight. Based on these results, 30min pre-treatment time point and dose of 20 mg/kg body weight of ML171 were selected for subsequent molecular and biochemical studies.

3.2. NOX activity and validation of ML171 action

NADPH oxidase is known to be involved in nociceptive processing via generation of intracellular superoxide anion. NOX activity was measured in terms of superoxide production in DRG (Fig. 3a) as well as in spinal cord (Fig. 3b) of formalin induced mice. Superoxide anion production was increasing up to 10 min of reaction time. NOX activity was significantly attenuated in case of ML171 pre-treatment as compared to DMSO treated group. As NOX1 is the main isozyme implicated in spontaneous pain, decrease in NOX activity is taken to represent NOX1 activity. Our results validate the inhibitory action of ML171 on NOX1 activation.

3.3. Effect of ML171 on ROS level

The variation pattern of ROS level followed the trend of NOX activity. Total level of ROS was found to be elevated after formalin injection at both peripheral (DRG) and central (spinal cord) sites (Fig. 4: a, b). There was an increase of approximately 77.3% in DRG and 55.8% in spinal cord of formalin induced mice as compared to saline treated ($p < 0.005$). NOX1 inhibitor ML171 brought down the ROS level significantly ($p < 0.005$) towards normal in both the tissues i.e. approximately 78.4% in DRG and 38.8% in spinal cord as compared to DMSO treated (FD) group.

3.4. Differential effect of ML171 on TNFR1 level in DRG and spinal cord

Release of tumor necrosis factor-alpha (TNF-α) following nerve or tissue injury sensitizes nociceptive neurons directly via TNFR1 (Li et al., 2004; Wheeler et al., 2014; Zhang et al., 2011). Therefore, the effect of ML171 administration was examined on TNFR1 level. Our results showed differential expression pattern of TNFR1 in tissue dependent manner (Fig. 5). A significant elevation (1.4fold, $p < 0.005$) was observed in the protein expression of TNFR1 at peripheral site (DRG) after formalin injection as compared to saline group (Fig. 5a), which was significantly inhibited by approx. 25% by ML171 administration ($p < 0.005$). However, there was no significant change observed in the expression of TNFR1 at central site (spinal cord) in all the four groups (Fig. 5b). The result suggests that propagation of pain in central nervous system is independent of TNFR1 mediated signaling.

3.5. Effect of ML171 on ERK1/2 phosphorylation in DRG and spinal cord

Noxious stimuli activate both C-fibers and Aδ-fibers of nociceptors leading to signal propagation via ERK1/2 activation. Therefore, phosphorylation of ERK1/2 was checked in terms of pERK1/2 in DRG as well as in spinal cord (Fig. 6). Formalin injection in the right hind paw resulted in the induced phosphorylation of ERK1/2 after 36–40 min in the ipsilateral side. The increase was approximately 1.32 folds, $p < 0.005$ in DRG (Fig. 6a) and 1.54 folds, $p < 0.005$ in spinal cord (Fig. 6b) of lumbar region (L3-L5). The elevated level of the phosphorylation of ERK1/2 was decreased significantly ($p < 0.005$) by approx. 22% in DRG and 49% in spinal cord, towards normal by ML171. Therefore, ability of ML171 to decrease pERK1/2/ERK1/2/β-actin ratio indicated its anti-nociceptive potential.

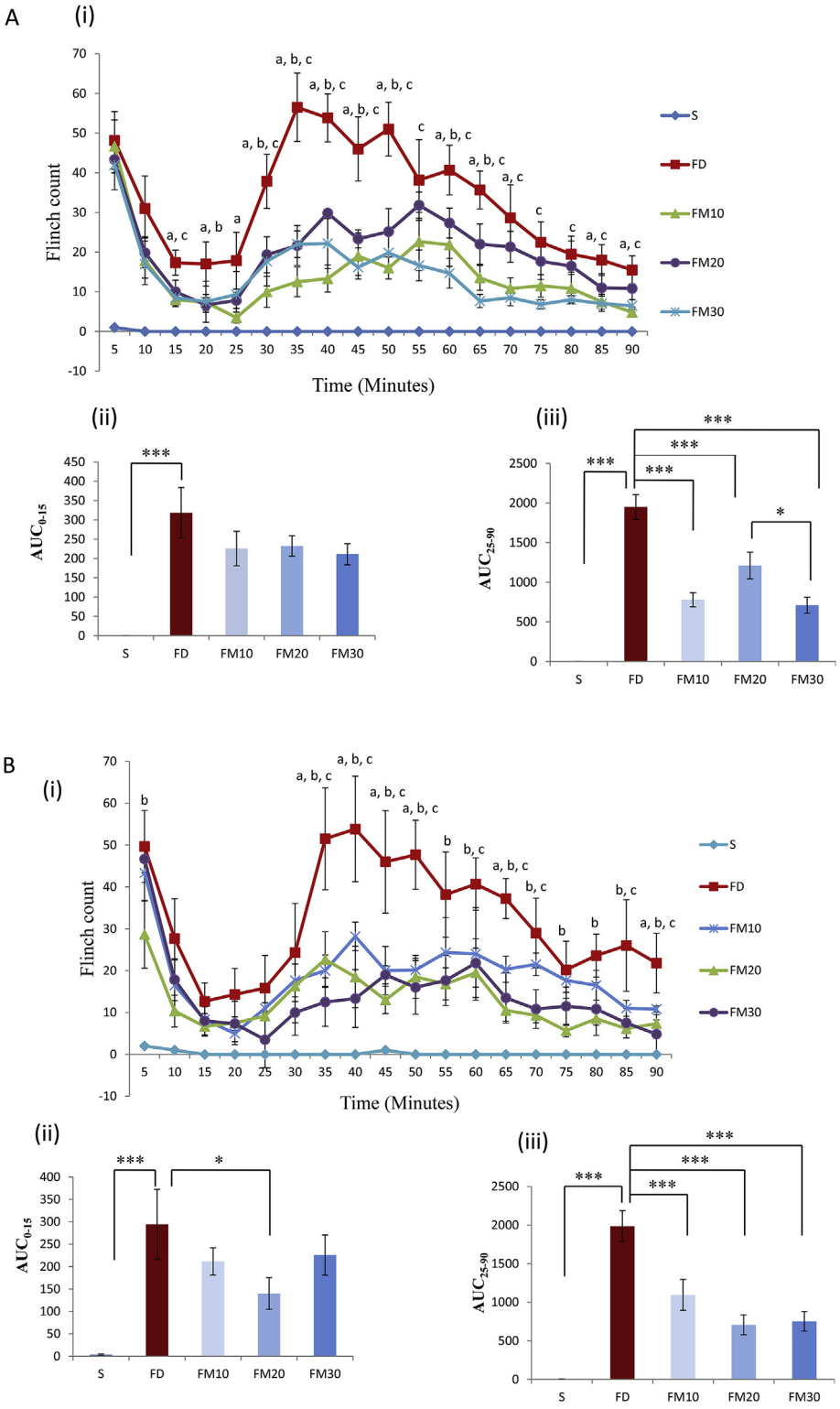

Fig. 1. Effect of ML171 on flinching behavior: The groups were classified as saline injected (S), formalin + DMSO treated (FD), formalin + ML171 treated with doses of 10 mg (FM10), 20 mg (FM20), and 30 mg (FM30) per kg body weight. DMSO and ML171 was administered (i.p.) immediately before the formalin injection (s.c.). Each group consists of 6 mice. **[A] Pre-treatment with ML171 immediately before the formalin injection. (i)** Paw flinching per 5min **(ii, iii)** Total area under curve for flinch count for phase1 from 0 to 15min ($AUC_{0\text{-}15}$), for phase2 from 25 to 90min ($AUC_{25\text{-}90}$) respectively **[B] Pre-treatment with ML171 30min before the formalin injection. (i)** Paw flinching per 5min **(ii, iii)** Total area under curve for flinch count for phase1 ($AUC_{0\text{-}15}$), for phase2 ($AUC_{25\text{-}90}$) respectively **[C] Pre-treatment with ML171 1hr before the formalin injection. (i)** Paw flinching per 5min **(ii, iii)** Total area under curve for flinch count for phase1 ($AUC_{0\text{-}15}$), for phase2 ($AUC_{25\text{-}90}$) respectively. Results represent means ± S.E.M. $^{a, b, c}$ denotes significant difference of groups FM10, FM20, and FM30 respectively with FD group ($p < 0.05$). *Denote significant difference between groups (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$).

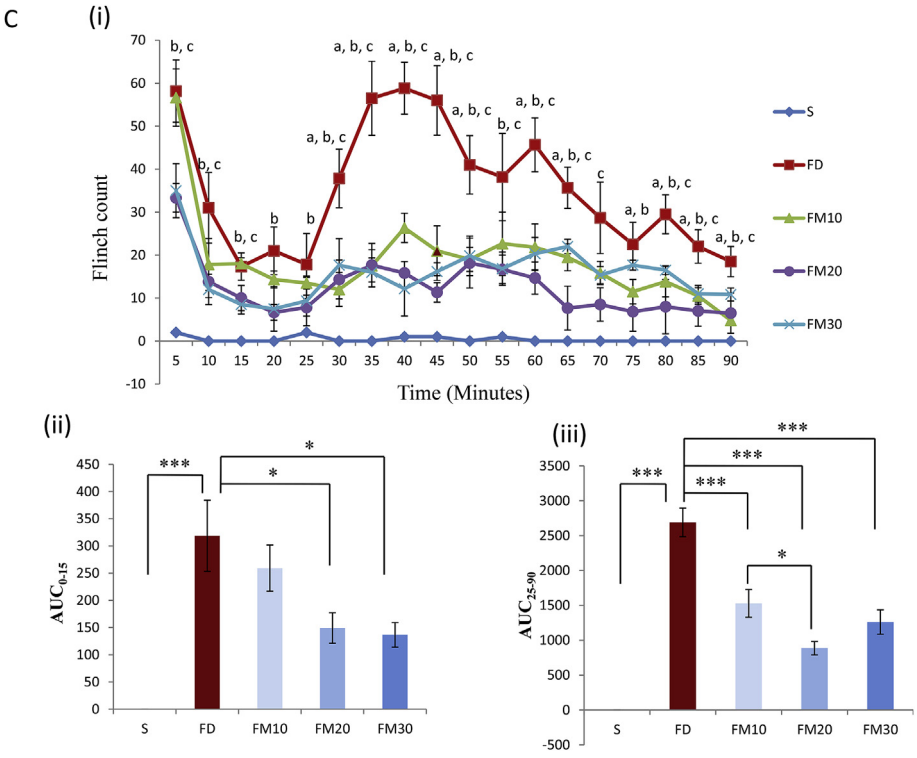

Fig. 1. (*continued*)

3.6. Expression of c-Fos in spinal cord

Expression of c-Fos in the dorsal horn of spinal cord is associated with nociceptive response and is considered as a neuronal marker of pain. The important role of c-Fos in the development of pain state is part of the adaptive response of spinal cord to continuous or subsequent nociceptive input or both. Further, c-Fos is the early gene expressed following ERK1/2 activation. Therefore, c-Fos expression was examined and compared in formalin induced and saline treated mice (Fig. 7). Increase in c-Fos expression was observed in ipsilateral dorsal horn of spinal cord of formalin induced mice as compared to saline group while it was declined after ML171 administration (Fig. 7a). The number of c-Fos positive cells in formalin induced mice was 25 ± 5 as compared to 9 ± 2 in saline treated group. ML171 administration decreased c-Fos positive cells up to 8 ± 3as compared to DMSO injected (FM) mice, $p < 0.005$ (Fig. 7b).

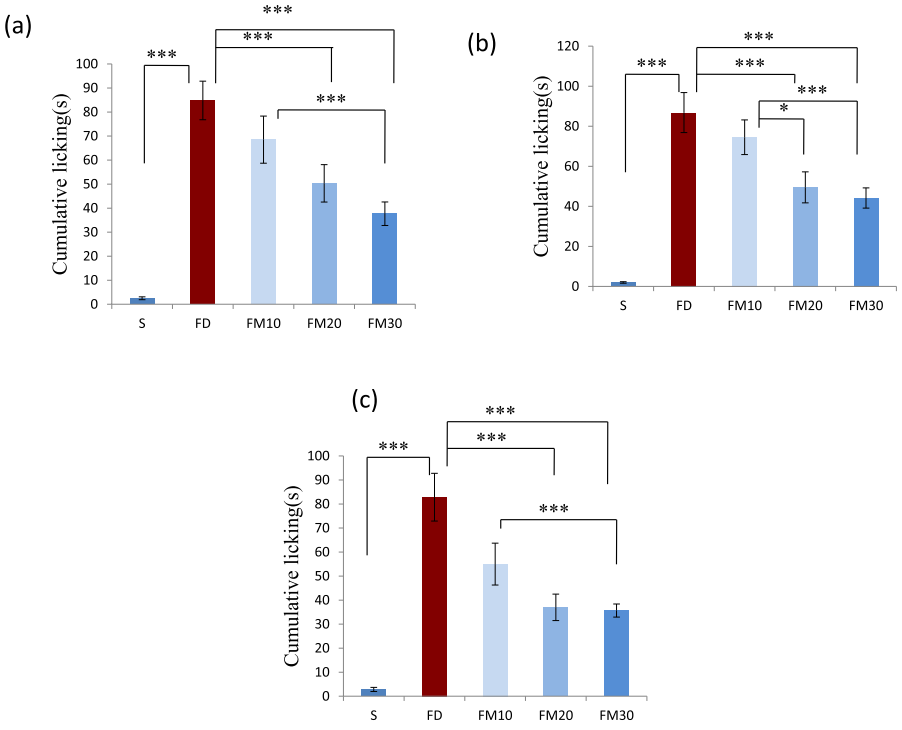

Fig. 2. Effect of ML171 on licking behavior: Animals were distributed in five groups (n = 6) as saline injected (S), formalin + DMSO treated (FD), formalin + ML171 treated with doses of 10 mg (FM10), 20 mg (FM20), and 30 mg (FM30) per kg body weight. Cumulative licking is represented at pre-treatment with ML171 **(a)** immediately before the formalin injection **(b)** 30min before the formalin injection **(c)** 1hr before the formalin injection. Results represent means ± S.E.M. *Denote significant difference between groups (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$).

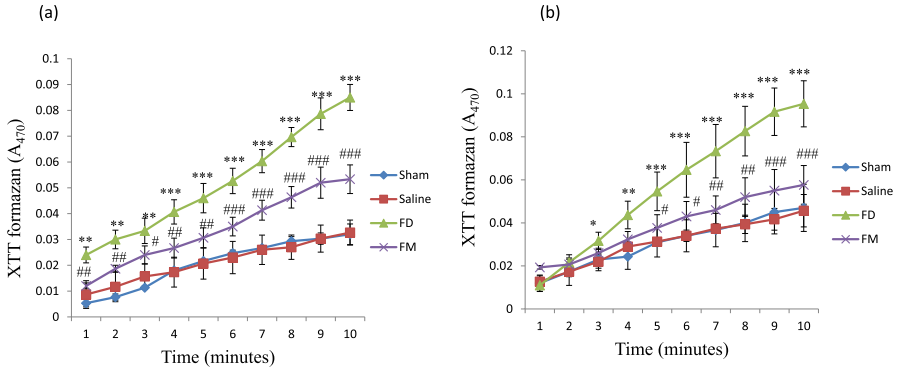

Fig. 3. Effect of ML171 on NOX1 activity: Mice were distributed in four groups (n = 5). The groups are classified as needle pricked (sham), saline injected (S), formalin + DMSO treated (FD), formalin + ML171 20 mg/kg BW (FM) **(a)** DRG. **(b)** Spinal cord. The activity was measured in terms of superoxide production by conversion of water-soluble XTT reagent to an orange-colored formazan product. Data are represented as XTT formazan (A_{470}). Results represent means ± S.E.M. *denotes significant difference as compared with saline group (*p < 0.05, **p < 0.01, and ***p < 0.005). #Denote significant difference as compared with FD group (#p < 0.05, ##p < 0.01, and ###p < 0.005).

4. Discussion

In current study we tested the anti-nociceptive effect of the newly identified NOX1 specific inhibitor ML171in mouse model of formalin induced acute nociception. Formalin induced mice is a valid and reliable model for assay of injury-produced inflammatory pain, useful for the screening of novel compounds (Tjolsen et al., 1992). Formalin test is a non reflexive test showing nociceptive behavior like spontaneous paw flinching/licking that occurs principally in two phases as early (first) and late (second) phase. The early phase lasts for 5–10 min, followed by a quiescent phase of approximately 5 min and a subsequent late phase up to 60–90 min. Early phase represents acute phase (non-inflammatory/neurogenic) and is suggested to be mediated primarily by direct chemical activation of local C-fibers and the late phase represents tonic phase (inflammatory) mediated via inflammatory mechanisms and sensitization within the dorsal horn of the spinal cord (Barrot, 2012). In the present study, NOX1 inhibitor ML171 is able to reverse formalin induced pain behavior. It resulted in reduced nociception in the first phase suggesting its inhibitory action on sensory afferent C-fiber activation. The second phase is the result of inflammatory input from the peripheral afferents and central sensitization via C-fibers entering the spinal dorsal horn. ML171 effectively inhibited second phase of formalin induced nociception i.e. spinal sensitization. Thus, the reduced pain behavior by ML171 in both phases is indicative of the attenuation of neurogenic as well as inflammatory pain. Our findings are supported by earlier report of neurogenic and inflammatory pain attenuation by antioxidant (Filho et al., 2008).

Inflammation following noxious stimulation is the consequential process for tissue repair. Notably, accumulating evidences indicate that ROS are involved in the sensitization of pain pathways (Gwak and Hulsebosch, 2012; Hackel et al., 2013). One of the most important sources of intracellular ROS is NADPH oxidase (NOX), a dedicated enzyme to ROS generation. Our knowledge about the role of NOX in pain is scanty. A few reports available only in the last decade suggest the contribution of NOX in nociceptive processing (Doyle et al., 2013; Ibi et al., 2008, 2011; Marone et al., 2018; Choi et al., 2013). The reports of Ibi et al. on NOX1 knockout mice suggest the role of NOX1 in capsaicin (Ibi et al., 2008) and morphine (Ibi et al., 2011) induced animal models. However, the mechanism of NOX1 mediated nociceptive signaling is still not worked out. The present study provides the insight about NOX1 mediated signaling mechanism in formalin induced nociception using NOX1 specific inhibitor ML171.

Significant attenuation was observed in flinching and licking behavior by NOX1 inhibitor ML171 in both phases which validates its anti-nociceptive action. This is in agreement with earlier reports showing involvement of NOX1 in inflammatory pain (Ibi et al., 2008). Further, the decrease in the activity of NOX by ML171 validates inhibitory action of ML171 on NOX1. Decrease in the activity of NOX is plausibly because of inhibition of NOX1 activity which is reflected as inhibition of superoxide production. The result confirms anti-nociceptive effect of ML171. Based on *in vitro* experiments in HEK293-Nox1 reconstituted cell system, Gianni et al. have suggested the possible mechanism of action of ML171 via specific inhibition of catalytic activity of NOX1. Additionally, they have shown that only over expression of NOX1 could overcome the inhibitory effect on superoxide formation by ML171 (Gianni et al., 2010).

Induced ROS level after formalin insult is observed which is in accordance with the earlier reports (Lu et al., 2012; Schwartz et al., 2009; Yamato et al., 2013). Further, ROS level and associated nociceptive effect was reported to be effectively inhibited after administration of free radical scavengers or superoxide dismutase mimetics (Schwartz et al., 2008; Yowtak et al., 2011) suggesting that ROS contributes to nociceptive processing. Antioxidants have been proposed to attenuate ROS induced pain signaling (Singh et al., 2018; Singh and Vinayak, 2015, 2017b). ROS generated via NOX1 have been reported to contribute to inflammation pain (Ibi et al., 2008). In our results the ROS production was attenuated after ML171 administration at peripheral (DRG) as well as at central site (spinal cord). The variation pattern in ROS level followed the trend of NOX activity. The functional correlation reflects that NOX1 is the major isozyme responsible for ROS production. ROS may activate NF-κB transcription factor which is crucial for development of inflammation. While NF-κB is ubiquitously expressed in a variety of cell types, its contribution during inflammatory and neuropathic pain is driven largely by signaling in the dorsal root ganglia and

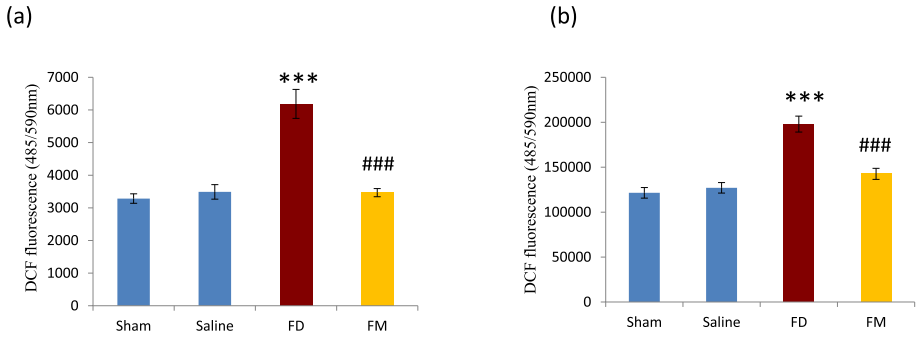

Fig. 4. Effect of ML171 on ROS level: Level of ROS in needle pricked (sham), saline injected (saline), formalin + DMSO treated (FD), formalin + ML171 20 mg/kg BW (FM). **(a)** DRG. **(b)** Spinal cord. ROS level was measured by oxidative conversion of H_2DCFDA to fluorescent DCF. Data are presented in terms of fluorescence intensity. Data are presented in terms of fluorescence intensity/mg protein. Each group included 5 mice. Results represent means ± S.E.M. *denotes significant difference as compared with saline group (*p < 0.05, **p < 0.01, and ***p < 0.005). #denotes significant difference as compared with FD group (#p < 0.05, ##p < 0.01, and ###p < 0.005).

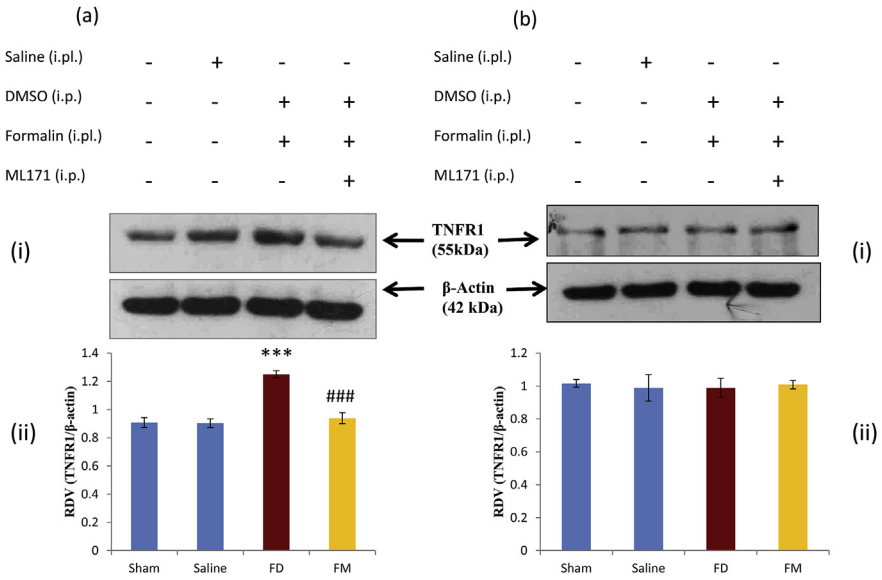

Fig. 5. Effect of ML171 on expression of TNFR1: (a) DRG (b) spinal cord in sham, saline injected (saline), formalin + DMSO treated (FD), formalin + ML171 20 mg/kg BW (FM). (a–i) and (b–i) show protein level measured by Western blot analysis. (a-ii) and (b-ii) represent densitometric analysis normalized by β-actin. Mice from each group (n = 5) were sacrificed after 40 min of formalin injection. Results represent means ± S.E.M. *denotes significant difference as compared with saline group (*p < 0.05, **p < 0.01, and ***p < 0.005). #denotes significant difference as compared with FD group (#p < 0.05, ##p < 0.01, and ###p < 0.005).

in astrocytes of spinal cord (Hou and Xu, 2018; Lee et al., 2011; Luo et al., 2014) via initiation of cytokine production (Chen et al., 2019; Chowdhury et al., 2019).

Pro-inflammatory cytokine TNF-α has been reported to sensitize nociceptive neurons indirectly via the induction of a proinflammatory cytokine cascade involving IL-1β, IL-6, and IL-8 which are released into the local environment following nerve or tissue injury (Chen et al., 2019; Singh and Vinayak, 2016). The reports suggest a direct effect of TNF-α on nociceptive pathway via TNFR1 (Li et al., 2004; Wheeler et al., 2014). Pain sensitization following formalin injection involves TNFR1 (Zhang et al., 2011). Proinflammatory cytokines mediate pain signaling via TNFR1 and lead to ERK1/2 activation (Singh and Vinayak, 2017a,b). Our finding of differential activation of TNFR1 at peripheral and central sites suggests that TNFR1 mediated ERK1/2 signaling by ROS is mainly operative in DRG, whereas propagation of pain in central nervous system is independent of TNFR1 mediated signaling. Other reports have also suggested the ROS mediated TNFR1 increase in mouse DRG neurons (Ma et al., 2009; Singh and Vinayak, 2017a). Further, recent findings from our lab suggest that ROS may activate ERK1/2 by an alternative signaling in spinal cord dorsal horn, mediated by Src family kinases (Singh and Vinayak, 2017a).

ERK1/2 activation appears to be closely correlated with pain-related behavior following noxious stimulus in peripheral and central sensitization (Zhang et al., 2014; Zhuang et al., 2005). Our result of ERK1/2 activation in terms of pERK1/2 level is in accordance with the above reports. Accumulating evidences suggest that pERK1/2 in dorsal horn neurons is required for the induction of central sensitization, which is behaviorally indicated in the second phase of pain response to formalin insult (Karim et al., 2001; Lee et al., 2012) and capsaicin-induced secondary mechanical allodynia (Kawasaki et al., 2004). pERK1/2 induces central sensitization via increasing the activity of excitatory glutamate receptors, AMPA and NMDA (Cao et al., 2012; Kohno et al., 2008). Activated pERK1/2 is translocated to the nucleus of dorsal horn neurons, activating the transcriptional factor CREB that is critical for long-term neuronal plasticity after noxious stimulation (Kawasaki et al., 2004; Shao et al., 2016), which leads to the transcription of various

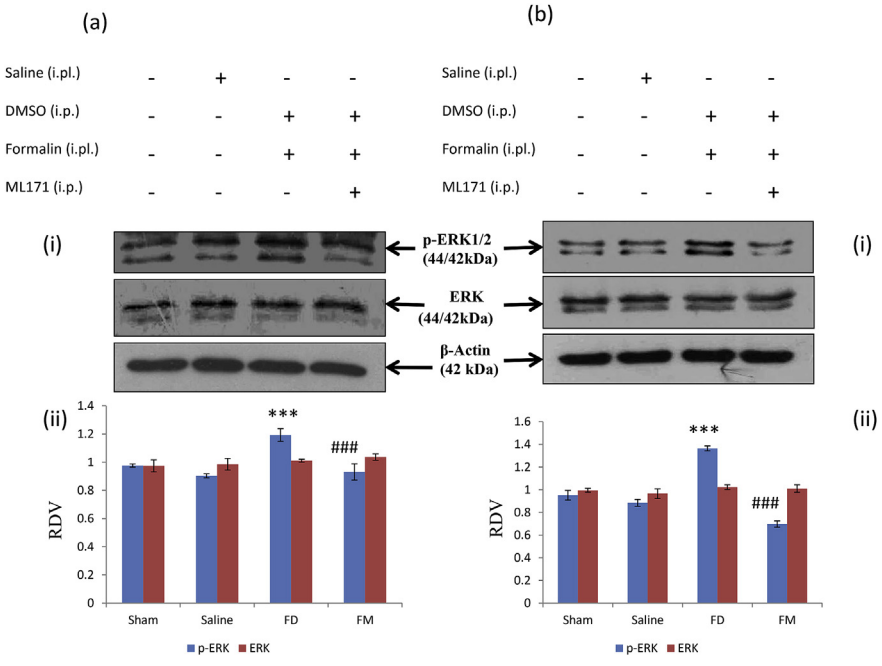

Fig. 6. Effect of ML171 on ERK phosphorylation: (a) DRG (b) spinal cord in sham, saline injected (saline), formalin + DMSO treated (FD), formalin + ML171 20 mg/kg BW (FM). (a–i) and (b–i) show protein level measured by Western blot analysis. (a-ii) and (b-ii) represent densitometric analysis normalized by β-actin. Mice from each group (n = 5) were sacrificed after 40 min of formalin injection. Results represent means ± S.E.M. *denotes significant difference as compared with saline group (*p < 0.05, **p < 0.01, and ***p < 0.005). #denotes significant difference as compared with FD group (#p < 0.05, ##p < 0.01, and ###p < 0.005).

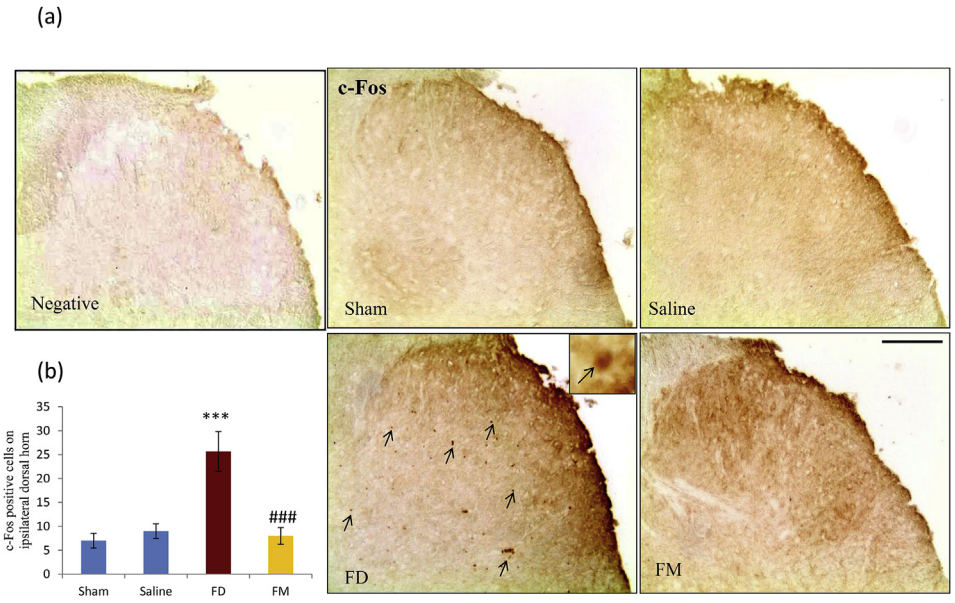

Fig. 7. Effect of ML171 on expression of c-Fos in dorsal horn of spinal cord: (a) Anti-nociceptive activity in terms of c-Fos expression in ipsilateral dorsal horn in sham, saline (S), formalin + DMSO treated (FD) and formalin + ML171 treated (FM) mice after 40 min of formalin injection. Arrows show c-Fos positive cells. Maginified image (40×) of c-Fos positive cell is shown as inset in FD group. Scale bar represents 200 μm. (b) c-Fos positive cells were considered irrespective of intensity of stain. c-Fos positive cells were counted manually in 4–6 sections for each group. Results represents means ± S.E.M. *denotes significant difference as compared with saline group (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.005$). $^{\#}$denotes significant difference as compared with FD group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.005$).

genes, i.e. c-fos, Cox-2, and NK-1, as well as other genes regulating synaptic plasticity after tissue injury and hypersensitivity (Ji, 2004). Further, the event of ERK1/2 activation was inhibited by attenuation of ROS by administration of NOX1 specific inhibitor ML171 in DRG as well as in spinal cord. Recent finding from our lab suggests that ERK1/2 activation is mediated by ROS which was inhibited by antioxidants (Singh and Vinayak, 2017b).

c-Fos is involved in signal transduction cascade that links extracellular events to intracellular adaptations. Expression of c-Fos is downstream to ERK1/2 activation. It is correlated with the onset and duration of noxious stimulus. We have observed increase in the number of c-Fos positive cells during formalin induced nociception which was lowered by ML171 administration. The c-Fos expression is confined to postsynaptic neurons of the spinal dorsal horn and serves as a useful marker of activated nociceptive neurons (Coggeshall, 2005; Harris, 1998; Hossaini et al., 2014; Hunt et al., 1987; Santos et al., 2018). Thus, c-Fos expression advocates for role of NOX1 induced ROS in pain sensitization.

5. Conclusion

This is the first report showing physiological action of ML171, a chemical inhibitor of NOX1. The overall result confirms anti-nociceptive action of ML171, mediated via inhibition of ROS production and ERK1/2 activation in formalin induced nociception.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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