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MODULATION OF TUMOUR NECROSIS FACTOR A ATTENUATES NALOXONE-PRECIPITATED OPIOID WITHDRAWAL SYNDROME

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ABSTRACT: The present study has been designed to investigate the effect of modulation of tumour necrosis α (TNF-α) on naloxone induced opioid withdrawal syndrome. Chronic morphine (5mg/kg,i.p) administration followed by a single injection of naloxone (8mg/kg, i.p) was used to precipitate opioid withdrawal syndrome in mice. Behavioral observations were made immediately after naloxone treatment. Withdrawal syndrome was quantitatively assessed in terms of withdrawal severity score and frequency of jumping, rearing, fore paw licking and circling. Administration of pirfenidone (200mg/kg, p.o) markedly and dose dependently attenuated naloxone induced morphine withdrawal syndrome. Thus, it is proposed that glial cells activation via toll like receptor (TLR) linked mechanism might be involved in the development of opioid dependence and precipitation of its withdrawal syndrome.

INTRODUCTION: Opioids have played critical role in achieving pain relief in both modern and ancient medicine. Yet, their clinical use can be limited secondary to unwanted side effects such as tolerance, dependence, reward and behavioral changes. Opioid addiction is a chronic, relapsing disorder. Left untreated, high morbidity and mortality rates are seen [1, 2]. Withdrawal syndrome, also called a discontinuation syndrome is a set of symptoms occurring on discontinuation or dosage reduction of some types of medications. . The opiate withdrawal syndrome emerges after repeated administration of heroin, morphine, and lasts for hours to a few days, depending upon the specific drug and the duration and dose of prior administration.

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In humans, the signs and symptoms of withdrawal include stomach cramps, diarrhea, rhinorrhea, sweating, elevated heart rate and increased blood pressure, irritability, dysphoria, hyperalgesia, and insomnia [3].

Animal models of "acute" (i.e., a single use) and "chronic" (i.e., long-term use) exposures have been useful in understanding opiate tolerance and dependence [4, 5]. Acute opiate exposure has been defined as the administration of an opiate in single or multiple dosages over 1 to 2 days, whereas chronic opiate exposure has been defined as administration of an opiate for a minimum of 5 days [6, 7]. In the preclinical setting the opiate withdrawal syndrome is well characterized in rodents. Both the precipitated and spontaneous withdrawal model is commonly used to study opiate withdrawal signs. Spontaneous opiate withdrawal occurs after the sudden discontinuation or the rapid tapering of chronically administered opiates.

Precipitated opiate withdrawal occurs after the use of an opioid receptor antagonist, which displaces the opioid receptor agonist. Both spontaneous and precipitated withdrawal syndromes are accompanied by symptoms of abstinence; however, the onset is faster with precipitated opiate withdrawal. Naloxone and Naltrexone are the most common competitive μ-opioid receptor antagonists used to precipitate opiate withdrawal[8]. Naloxone is a drug used to counter the effects of opiate overdose, for example heroin or morphine overdoses. It has an extremely high affinity for μ-opioid receptors in the central nervous system. Its rapid blockade of those receptors often produces rapid onset of withdrawal symptoms.

Extensive research is going on for the development of non-addicting opioids and/or agents that can prevent or reverse the addiction processes such as methadone, a long-acting opiate, which can also be used for detoxification from opiate medications. It is effective in reducing symptoms of opiate withdrawal especially for intravenous opiate users. Clonidine, a non-opiate alpha-2 agonist, decreases sympathetic outflow to the body. This can often reduce the symptoms of opiate withdrawal. However, none of the available options promises to conclusively treat the condition of opioid dependence and its related abstinence syndrome [9].

Opiate drugs exert their effects by binding to three opioid receptor types (μ, γ, and κ) and mimicking the actions of endogenous opioid peptides, the endorphins, endomorphins, enkephalins, and dynorphins. The μ opioid receptor (MOR) subtype is critical for the rewarding effects of heroin and morphine. The most prominent neuroadaptive changes during morphine induced dependence include desensitization of MORs and upregulation of the cAMP pathway. The mitogen activated protein kinase (MAPK) pathway as well as Ca2+ signaling are also affected during morphine dependence. The primary consequence of morphine withdrawal is 'superactivation' of adenylyl cyclase (AC) and a subsequent overproduction of its downstream signaling molecule, cAMP. Other cAMP actions during withdrawal include PKA-mediated enhanced GABAergic synaptic transmission in areas such as periaqueductal grey (PAG), ventral tegmental area (VTA), nucleus accumbens (NAcc.) and dorsal raphe [10, 11].

Among the brain regions implicated in opiate dependence and withdrawal, the periaqueductal gray area (PAG) appears to be critical in regulating the complex signs and symptoms of opioid withdrawal. Numerous neurochemical mechanisms in the PAG have been identified that may contribute to the opioid withdrawal syndrome[12]. Other receptors like glutamate, mucarinic, nicotinic and toll like receptors are also involved in morphine withdrawal syndrome.

Toll like receptors play important role in morphine wihdrawal syndrome in which glial cells, particularly astrocytes, envelop neuronal synapses and participate in the physiological control of synaptic transmission and plasticity via the release of synaptically effective mediators, a process called gliotransmission [13, 14]. Morphine binds to an accessory protein of glial toll-like receptor 4 (TLR4), myeloid differentiation protein 2 (MD-2), thereby inducing TLR4 oligomerization and triggering pro-inflammatory cytokines [15]. Direct activation of glial TLR4 induces overexpression of TNF*α* [16, 17]. Morphine withdrawal induces astrocytic activation to release TNF*α* in the PAG. It has also been proved that exogenous TNF*α* injection into the PAG evokes morphine withdrawal-like behaviors [18].

Pirfenidone (5-methyl-1-phenyl-2-(1H)-pyridone) is an orally active small molecule comprising a modified phenyl pyridone. The compound exhibits both anti-inflammatory and antifibrotic activities that have been reported both in vitro and in vivo. Pirfenidone has been clinically evaluated for its safety and efficacy in numerous disorders such as multiple sclerosis, primary sclerosing cholangitis, chronic hepatitis C, myelofibrosis, neurofibro-mitosis, and fibrotic renal disorders. In Multiple sclerosis (MS), a demyelinating disorder characterized by neurological deficits, demyelinating lesions and progressive axonal loss in the white matter, there is involvement of TNF-α in the demyelination and consequently in the progressive pathogenesis of Multiple sclerosis. Pirfenidone suppressed the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) by a translational mechanism, which was independent of activation of the mitogen-activated protain kinase (MAPK) 2, p38 MAP kinase, and c-Jun N-terminal kinase (JNK) [19].

These findings from previous studies suggest that pirfenidone has not merely an anti- fibrotic effect but also an anti-inflammatory effect via cytokine regulation (TNF-α) and provides a clinical effect. The most extensive clinical studies of pirfenidone are for treatment of idiopathic pulmonary fibrosis (IPF), a chronic interstitial lung disease [20]. Together, these observations suggest that pirfenidone might be a useful therapeutic for several different diseases. So the present study has been designed to study the effect of modulation of TNF-α by pirfenidone on morphine withdrawal syndrome.

Materials and Methods: Swiss albino mice, weighing 20-30 grams were employed in the present study (procured from Central Research Institute (CRI), Kasauli). They were maintained on standard laboratory diet (Aashirwaad feeds Ltd., Chandigarh, India) and tap water ad libitum. They were housed in the animal house of Rayat and Bahra Institute of Pharmacy (RBIP), Sahauran and were exposed to natural photoperiod. The experiments were conducted in a semi sound proof laboratory between 5:00 am to 5:00 pm. The experimental protocol of the study was duly approved by Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Reg. No. 1380/a/10/CPCSEA)

Drugs and Chemicals: All the drug solutions were freshly prepared before use. Pirfenidone was used as a test drug and was obtained from Cipla Ltd, Malpur, Dist. Solan, India. Morphine was purchased from Jackson Laboratories, Amritsar, India. Naloxone was purchased from Samarth Life Sciences Pvt. Ltd, Solan, India. All the reagents used in this study were of analytical grade. Morphine and Naloxone were dissolved in sterile saline (0.9% w/v NaCl). Pirfenidone available as powder was suspended in 0.5% w/v Carboxy Methyl Cellulose (CMC) to get a final concentration. Morphine and Naloxone were injected intraperitoneally (i.p.). Pirfenidone was administered orally with the help of an oral tube (cannula).

Induction of morphine withdrawal syndrome in mice: Morphine was administered (5 mg/kg, i.p.) twice daily for a period of 5 days. On the sixth day, 2 h after the injection of morphine, an injection of naloxone (8mg/kg, i.p.) was given in order to precipitate withdrawal syndrome in mice. Behavioral observations were made in two phases immediately after injecting naloxone (Way *et al*., 1969; Rehni and Singh 2011a, 2011b). The observations were made in a transparent perspex observation chamber with dimensions of 30cm×30cm×30cm. Two observers simultaneously observed each animal for all of the withdrawal measures, and the mean value of both observations was recorded.

Initial 30-min observation period immediately after naloxone administration was the segment of observation period that was used to assess withdrawal severity score and jumping frequency in mice, while the second 30-min observation period (started after the completion of the earlier observation period) represented the segment of observation period that was used to assess the frequency of rearing, fore paw licking and circling. The morphine/vehicle injections were given at 5:00 a.m. and 5:00 p.m. daily from day 1 to day 5. On the last day of the protocol (day 6), naloxone/vehicle was injected at 07:00 a.m. (2 h after the last morphine injection at 5:00 a.m.). However, Pirfenidone/vehicle was administered at 6:00 a.m. daily from day 1 to day 5.

Assessment of morphine withdrawal syndrome in terms of jumping frequency in mice: Repeated jumping behavior precipitated by opioid antagonist naloxone has been considered as a predominant sign for quantification of morphine withdrawal syndrome in mice (Way *et al*., 1969; Rehni, 2011a, b). Jumping frequency noted in the first phase of observation period (the initial 30min) was used as a quantitative measure of morphine withdrawal

Assessment of morphine withdrawal syndrome in terms of withdrawal severity score in mice: Withdrawal severity score was employed to evaluate the magnitude of withdrawal syndrome in mice in terms of behavioural parameters, viz., fore paw tremor, wet dog shake, straightening, ptosis and sneezing in a composite manner (Shaw-Lutchman *et al*., 2002; Inoue *et al*., 2003; Liu *et al*.,

2007; Rehni *et al*., 2008a, b). The severity of opioid withdrawal phenomenon was graded on a scale of 0–15 (normal score, 0; maximal withdrawal severity score, 15). In each of the animal behavioural aspect of severity scores of withdrawal, 0 score was awarded for no change in the normal behaviour of mice with respect to each observation criterion, 1 score was awarded for a mild increase in the respective observation criteria, 2 score was awarded for a moderate increase in the respective observation criterion and 3 score was awarded for a severe increase in the respective observation criterion.

Thus, the higher the score, the more severe is the withdrawal syndrome. The test was performed immediately after naloxone administration, and the results were based on observations spanning 30 min and global score depicting both the general severity of the feature in terms of magnitude of a given episode as well as its frequency during the observation period. The cumulative withdrawal severity score was obtained by adding scores awarded to each of the five behavioral aspects, i.e. fore paw tremor, wet dog shake, straightening, ptosis and sneezing in a composite manner.

Assessment of morphine withdrawal syndrome in terms of rearing, fore paw licking and circling frequency in mice: Rearing, fore paw licking and circling frequency observations were made during the 30 min observation period as a measure of the severity of behavioral aberration ascribed to experimental withdrawal phenomenon. These parameters have been noted to be indicative of the intensity of withdrawal syndrome. (Glick, 1976; Patkina, 1978; Falls, 1989).

Assessment of the effect of test drugs on locomotor activity using an actophotometer: The locomotor activity was monitored by using actophotometer. The actophotometer employed to assess the locomotor activity in mice had a test chamber of the dimensions 18 inch (length) × 18 inch (width) × 12 inch (height). Two of the adjoining lateral walls of the test chamber consisted of six sources of light at a lower level (1 inch from base) and six sources of light at a higher level (2 inch from base). Other two walls consisted of photo-cell-based receptors, present opposite to each source, receiving the light. Therefore, the total number of light beams assessing the movement of the mice was 24. Each time an animal interrupted the light, the same was recorded by the apparatus. For locomotor activity trial, animals were individually placed in the activity meter, and total activity count was registered for every 10 min until the completion of a 90-min observation period. The locomotor activity of each animal was expressed in terms of total photobeam interruptions per 10 min (Reddy, 1998). The locomotor activity in mice was assessed immediately before and for a 90-min period after the injection of test drug, and the period for each trial was 10 min.

Experimental Design: Five Groups were employed in the present study and each group consisted of five Swiss albino mice. Dosage of the drugs was selected on the basis of the previous reports (Rehni et al., 2012)

Group I:

Vehicle -Vehicle Control: Mice were administered with vehicle of morphine (normal Saline, 10 ml/kg, i.p) twice daily for a period of 5 days (Day1 to Day 5). Vehicle (CMC, 10ml/kg, i.p) for pirfenidone was simultaneously injected once daily for the same period of 5 days. Vehicle (normal saline 10ml/kg, i.p) for naloxone was then injected in the morning of day 6, 2 h after administering vehicle (normal saline, 10ml/kg, i.p) for morphine.

Group II:

Vehicle – Naloxone control: Mice were administered with vehicle of morphine (normal saline, 10ml/kg, i.p) twice daily for a period of 5 days (Day 1 to Day 5). Vehicle (CMC, 10ml/kg.i.p) for pirfenidone was simultaneously injected once daily for the same period of 5 days. Naloxone (8mg/kg, i.p) was then injected in the morning of day 6, 2 h after administering vehicle (normal saline, 10ml/kg, i.p) for morphine.

Group III:

Morphine - Naloxone control: Morphine (5mg/kg, i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5). Vehicle (CMC, 10 ml/kg, i.p) for pirfenidone was simultaneously

injected once daily for the same period of 5 days. Naloxone (8mg/kg, i.p) was then injected in the morning of day 6, 2 h after administering morphine (5mg/kg,i.p)

Group IV:

Pirfenidone treatment + Morphine - Naloxone treated group: Morphine (5mg/kg, i.p) was administered twice daily for a period of 5 days (Day 1 to Day 5) Pirfenidone (200mg/kg, p.o) simultaneously injected once daily for the same period of 5 days. Naloxone (8mg/kg, i.p) was then injected in the morning of day 6, 2 h after administering morphine (5 mg/kg, i.p)

Group V:

Pirfenidone (per se locomotor activity test group): After recording the basal reading locomotor activity count, each mouse was administered pirfenidone (200mg/kg), and locomotor activity count was repeated 30 min after dosing.

TABLE 1: *IN VIVO* MORPHINE WITHDRAWAL PROTOCOL

		GROUP I Vehicle-Vehicle treated	GROUP II Vehicle-Naloxone treated	GROUP III Morphine-Naloxone treated	GROUP IV Pirfenidone+Morphine-Naloxone treated
1st day	5:00 am	V1	V1	M	M
	6:00 am	V2	V2	V2	P
	5:00 pm	V1	V1	M	M
2nd day	5:00 am	V1	V1	M	M
	6:00 am	V2	V2	V2	P
	5:00 pm	V1	V1	V1	M
3rd day	5:00 am	V1	V1	M	M
	6:00 am	V2	V2	V2	P
	5:00 pm	V1	V1	V1	M
4th day	5:00 am	V1	V1	M	M
	6:00 am	V2	V2	V2	P
	5:00 pm	V1	V1	V1	M
5th day	5:00 am	V1	V1	M	M
	6:00 am	V2	V2	V2	P
	5:00 pm	V1	V1	V1	M
6th day	5:00 am	V1	V1	M	M
	7:00 am	V1	N	N	N

V1 represents vehicle for morphine (normal saline, 10ml/kg. i.p) and naloxone (normal saline, 10ml/kg, i.p), V2 represents vehicle for pirfenidone (carboxymethylcellulose, 10ml/kg, i.p), M represents morphine (5mg/kg, i.p), N represents the naloxone treatment (8mg/kg, i.p), P represents the pirfenidone treatment (200mg/kg, p.o)

TABLE 2: LOCOMOTOR TEST PROTOCOL

GROUP V	Pirfenidone per se locomotor activity
Before	Basal behavioral trial
10:00 am	Pirfenidone (200mg/kg, p.o)
10:30 am	Test behavioral trial

Statistical analysis:- The observations were statistically analyzed with the help of InStat3. All of the results were expressed as mean ± standard error of the mean (SEM).

Results were analyzed using one way analysis of variance (ANOVA), followed by Tukey's multiple comparsion test and $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION:

Effect of pirfenidone on naloxone-induced withdrawal syndrome in mice in terms of jumping, circling, fore paw licking, rearing and withdrawal severity score behavioral parameters: Administration of morphine (5mg/kg, i.p) twice daily for a period of 5 days followed by a single injection of naloxone (8mg/kg, i.p) precipitated withdrawal syndrome in mice as reflected by a significant increase in stereotyped jumping behavior, withdrawal severity score, rearing activity, fore paw licking behavior and circling behavior in morphine-naloxone control group, when compared to that of vehicle treated

control groups. These withdrawal symptoms were precipitated by administration of naloxone. Naloxone is the most common competitive μ-opioid receptor antagonists used to precipitate opiate withdrawal.

It is used to counter the effects of opiate overdose, for example heroin or morphine overdose. It has an extremely high affinity for μ-opioid receptors in the central nervous system and causes rapid blockade of opioid receptors which often produces rapid onset of withdrawal symptoms (**Tables 3, 4, 5, 6, 7**) (**Figure 1, 2, 3, 4, 5**).

The behavioral effects seen in the pirfenidone treated animals were significantly higher than the effect produced by each of the vehicle treatment groups but administration of pirfenidone significantly attenuated the propagation of morphine dependence as compared to morphine naloxone control group and it thereby reduced withdrawal signs reflected by decrease in all the behavioral parameters studied in morphine dependent mice (Tables 3, 4, 5, 6, 7) (Figure 1, 2, 3, 4, 5).

Previous studies have shown that pirfenidone significantly and dose dependently reduced the formation of intracellular reactive oxygen species (ROS) and tumour necrosis factor-α (TNF- α) in various diseases. Therefore, it might be postulated that this decrease in the intensity of the naloxone precipitated withdrawal syndrome by pirfenidone may be due to reduced level of TNF-α which is involved in the precipitation of opioid withdrawal syndrome via TLR 4 and glial cells activation induced by chronic morphine administration followed by a naloxone challenge.

TABLE 3: EFFECT OF PIRFENIDONE ON JUMPING FREQUENCY

Groups	Treatment	Jumping frequency (sec)
I	Vehicle-Vehicle control	1.6±0.2449
II	Vehicle-Naloxone control	1.2±0.2831
III	Morphine-Naloxone control	16.8±1.241
IV	Pirfenidone treatment + morphine-naloxone	11.4±1.030

For all groups *n*=5, all the data for jumping frequency are represented as mean ± standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

TABLE 4: EFFECT OF PIRFENIDONE ON CIRCLING FREQUENCY

Groups	Treatment	Circling frequency (sec)
I	Vehicle-Vehicle control	0.6±0.2449
II	Vehicle-naloxone control	0.8±0.2228
III	Morphine-naloxone control	10.8±1.020
IV	Pirfenidone treatment + morphine-naloxone	6±0.6325

For all groups *n*=5, all the data for circling frequency are represented as mean ± standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

TABLE 5: EFFECT OF PIRFENIDONE ON FORE PAW LICKING

Groups	Treatment	Fore paw licking
I	Vehicle-Vehicle control	1±0.3162
II	Vehicle-Naloxone control	1.4±0.3148
III	Morphine-Naloxone control	15±0.6325
IV	Pirfenidone treatment + morphine – naloxone	11.4±0.8124

For all groups *n*=5, all the data for fore paw licking are represented as mean ± standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

TABLE 6: EFFECT OF PIRFENIDONE ON REARING FREQUENCY

Groups	Treatment	Rearing Frequency
I	Vehicle-Vehicle control	0.6±0.2449
II	Vehicle-naloxone control	0.7±0.2585
III	Morphine- naloxone control	20.4±2.040
IV	Pirfenidone treatment + morphine-naloxone	13.8±1.356

For all groups *n*=5, all the data for rearing frequency represented as mean ± standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

TABLE 7: EFFECT OF PIRFENIDONE ON WITHDRAWAL SEVERITY SCORE (WSC)

Groups	Treatment	WSC (sec)
I	Vehicle – Vehicle control	0.8±0.2000
II	Vehicle – naloxone control	1.0±0.4000
III	Morphine – naloxone control	14.6±0.6000
IV	Pirfenidone + morphine – naloxone treated	11.8±0.3742

For all groups *n*=5, all the data for WSC are represented as mean ± standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

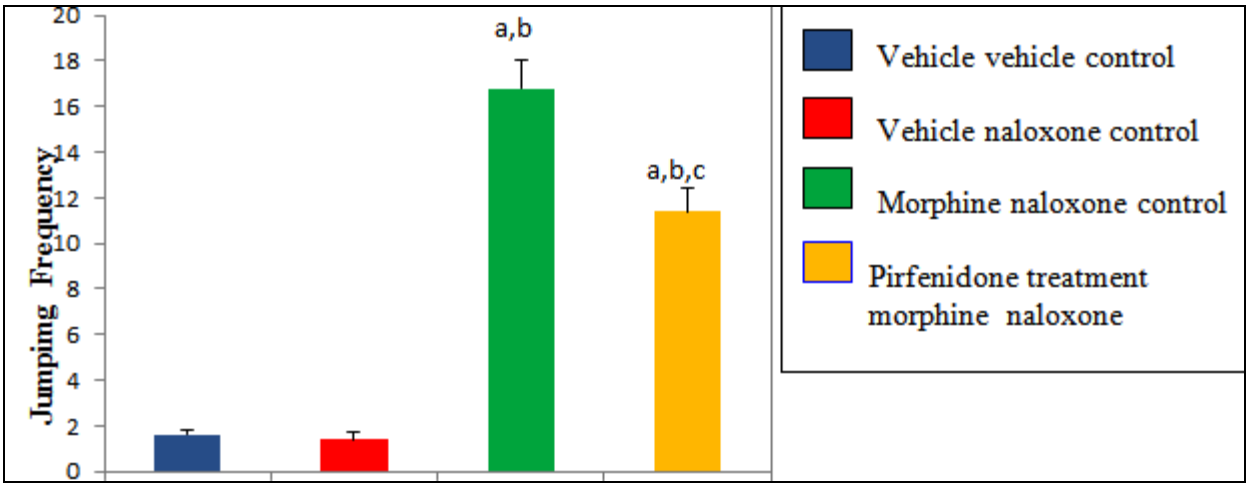

FIGURE 1: EFFECT OF PIRFENIDONE ON NALOXONE INDUCED WITHDRAWAL SYNDROME IN MICE REPRESENTED IN TERMS OF JUMPING FREQUENCY. For all groups (n=5) values are represented as mean±standard error mean (SEM) and were statistically analysed using one way ANOVA followed by Tukey's multiple comparison test. a= P<0.05 versus vehicle-vehicle control, b= P<0.05 versus vehicle-naloxone control, c=P<0.05 versus morphine-naloxone control

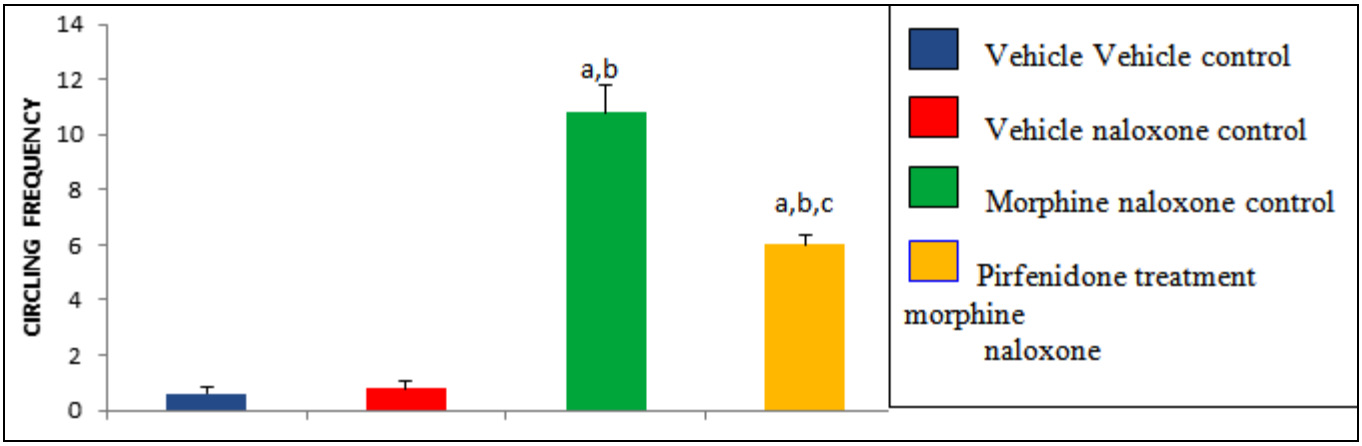

FIGURE 2: EFFECT OF PIRFENIDONE ON NALOXONE-INDUCED WITHDRAWAL SYNDROME IN MICE REPRESENTED IN TERMS OF CIRCLING BEHAVIOR. For all groups (n=5) values are represented as mean±standard error mean (SEM) and were statistically analysed using one way ANOVA followed by Tukey's multiple comparison test. a= P<0.05 versus vehicle-vehicle control, b= P<0.05 versus vehicle-naloxone control, c=P<0.05 versus morphine-naloxone control

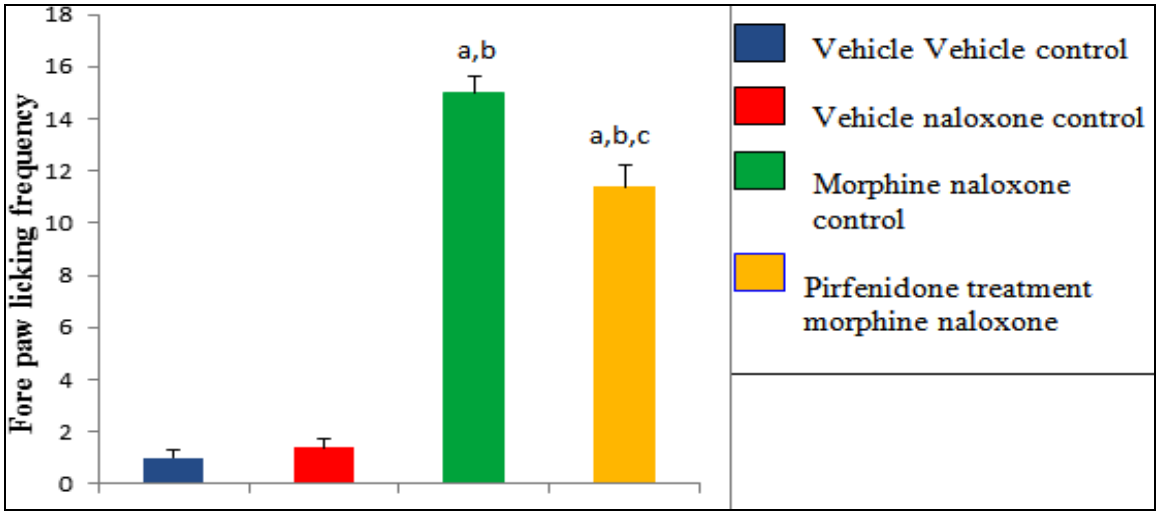

FIGURE 3: EFFECT OF PIRFENIDONE ON NALOXONE-INDUCED WITHDRAWAL SYNDROME IN MICE REPRESENTED IN TERMS OF FORE PAW LICKING. For all groups (n=5) values are represented as mean±standard error mean (SEM) and were statistically analysed using one way ANOVA followed by Tukey's multiple comparison test. a= P<0.05 versus vehicle-vehicle control, b= P<0.05 versus vehicle-naloxone control, c=P<0.05 versus morphine-naloxone control

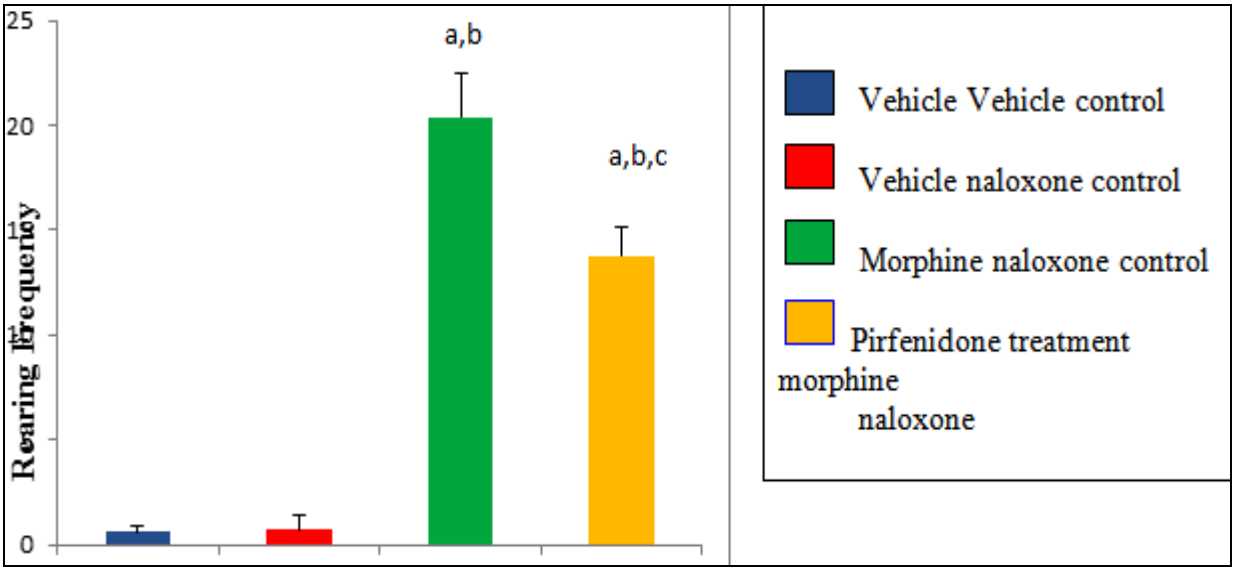

FIGURE 4: EFFECT OF PIRFENIDONE ON NALOXONE-INDUCED WITHDRAWAL SYNDROME IN MICE REPRESENTED IN TERMS OF REARING FREQUENCY. For all groups (n=5) values are represented as mean±standard error mean (SEM) and were statistically analysed using one way ANOVA followed by Tukey's multiple comparison test. a= P<0.05 versus vehicle-vehicle control, b= P<0.05 versus vehicle-naloxone control, c=P<0.05 versus morphine-naloxone control

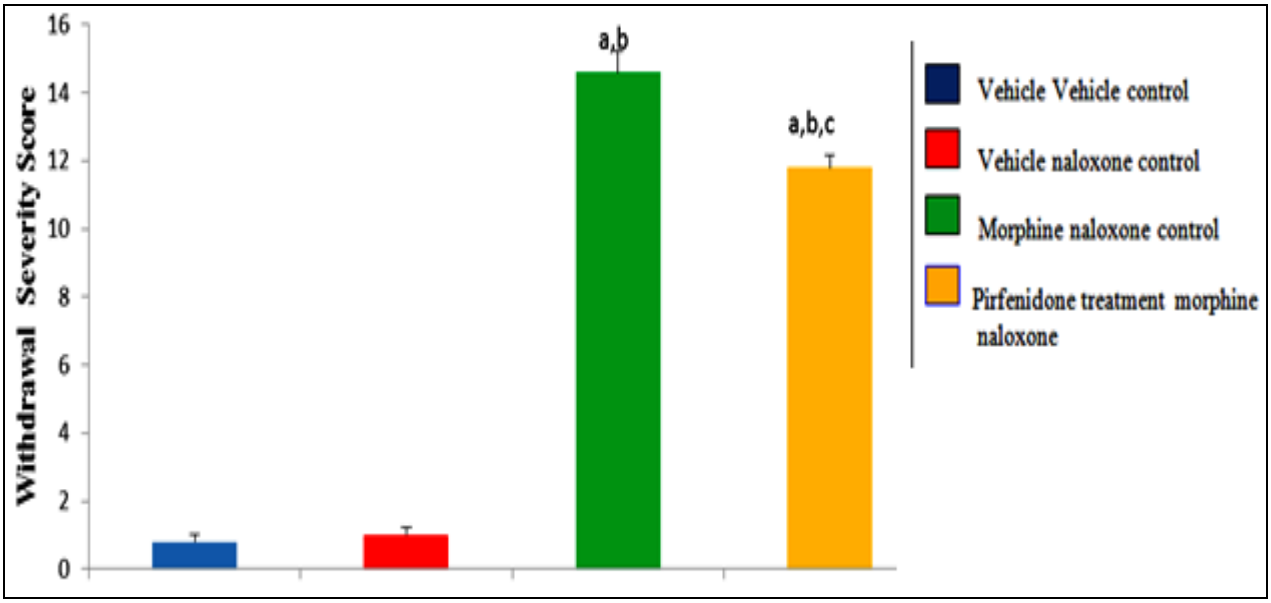

FIGURE 5: EFFECT OF PIRFENIDONE ON NALOXONE INDUCED WITHDRAWAL SYNDROME IN MICE REPRESENTED IN TERMS OF WITHDRAWAL SEVERITY SCORE. For all groups (n=5) values are represented as mean±standard error mean (SEM) and were statistically analysed using one way ANOVA followed by Tukey's multiple comparison test. a= P<0.05 versus vehicle-vehicle control, b= P<0.05 versus vehicle-naloxone control, c=P<0.05 versus morphine-naloxone control

Effect of pirfenidone on locomotor activity: A sedative test compound if assessed for a potential effect on opioid withdrawal syndrome in mice, may give false positive results by generalized suppression of a multitude of behavioral activities inspite of having no effect on the biochemical progression of opioid dependence. Therefore, the experimental design analyzing the locomotor activity was aimed at assessing the potential sedative effect of the test compounds. Thus, the locomotor activity test was performed with a view of affirming the potential effect of the test drugs on the general status of CNS excitation in animals employed in present study.

Administration of pirfenidone *per se* did not exert any sedative effect on the central nervous system as measured in terms the locomotor activity count (**Table 8**) (**Figure 6**).

TABLE 8: EFFECT OF PIRFENIDONE ON LOCOMOTOR ACTIVITY IN MICE

Test Drug	Locomotor Activity	
	Before Dosing	After Dosing
Pirfenidone (200mg/kg, p.o)	178.3±2.048	165.2±2.341

For all groups *n*=5, all the data for locomotor activity are represented as mean ± standard error of the mean (SEM), and were statistically analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

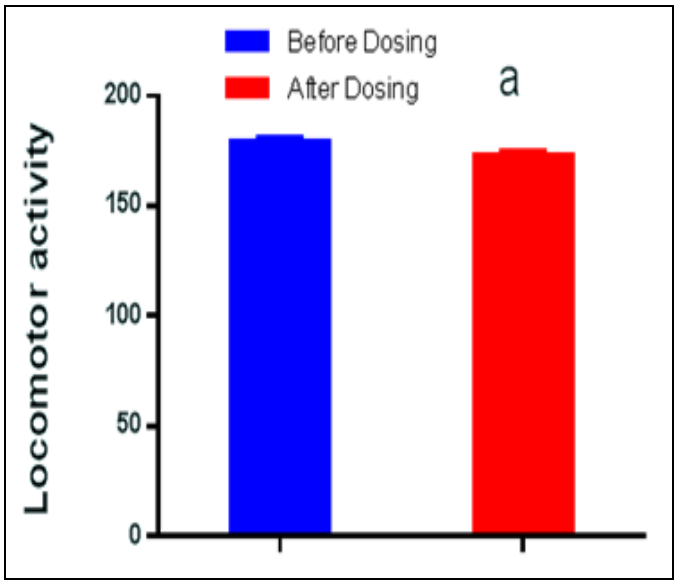

FIGURE 6: EFFECT OF PIRFENIDONE ON LOCOMOTOR ACTIVITY IN MICE. [a]P<0.05 vs locomotor activity count data assessed before dosing.

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