

The effect of methadone and haloperidol combination on anxiety induced by morphine withdrawal in male mice

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ABSTRACT

Background and Objective: Regarding inefficiency of common drugs used for alleviation of anxiety due to narcotics withdrawal, the present study was evaluated methadone and haloperidol co-drugs therapy on anxiety due to morphine withdrawal.

Materials and Methods: Ninety eight NMRI male mice were divided into acute and chronic experimental groups. Then, each group was divided into 7 subgroups: saline, morphine (control), methadone, haloperidol, methadone+haloperidol, methadone+haloperidol with 2/1 and 1/2 ratio, respectively. Mice were addicted chronically (over 8 days) by receiving escalating doses of morphine and acute (morphine was applied only on 8th day) procedures. Anxiety was induced by naloxone application in addicted mice. Elevated plus-maze and open field tests were used for evaluation of anxiety.

Results: Obtained data showed that in both chronic and acute groups, treatment with co-drugs methadone and haloperidol could markedly alleviate anxiety signs produced by interruption of morphine consumption.

Conclusion: We found out that the anxiety as a major sign of morphine withdrawal sign could be diminished by methadone+haloperidol therapy versus drugs alone.

1. Introduction

It is well established that long-term administration of morphine could produce tolerance and dependence (1) and anxiety as a prominent withdrawal sign occur due to morphine withdrawal (2). Regarding many researches on opioid use, so far no main mechanisms behind tolerance, dependence, and withdrawal syndrome have been known. The neurotransmitters like nitric oxide, dopamine and glutamate could mediate mentioned signs (3-5). Also, the existence of a mutual connection between dopamine secretion and NO production and the

role of NO as an anxiety modulator have been shown (6, 7). Nonetheless, an increase in NO production is associated with anxiety elevation (8).

Methadone therapy is currently known to be the most appropriate method of morphine detoxification. Methadone is an opioid and NMDA receptor antagonist (9). Some studies have shown that numerous patients under maintenance treatment with methadone are diagnosed with psychological disorders such as anxiety and depression (10).

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Haloperidol is used for treatment of psychological disorders such as schizophrenia, mania, and psychosis and is a butyrophenone anti-psychotic medication. This drug is a dopamine antagonist and has tendency to D₂ dopamine receptors (11). Researches have shown this medication prevents NO production by inhibition of calcium calmodulin protein kinase II (CaMkII), and therefore can potentially decrease anxiety withdrawal sign (12). Moreover, some reports have shown that anti-psychotic medications (*in vitro*) are potent to prevent nitric oxide synthetase by inhibition of CaMkII and therefore, reduce anxiety (13). On the other hand, other studies have shown that some anti-psychotic medications reduce anxiety and fear by blocking D₂ dopamine receptors (14, 15).

According to mentioned reports and regarding occurrence of anxiety following morphine withdrawal, the effect of combined methadone and haloperidol therapy on anxiety induced in male morphine-addicted rat was investigated in the present study.

2. Materials and Methods

2.1 Animals

This experimental study was performed on 98 NMRI male mice (Razi Institute, Iran; 20-25 grams). The mice were kept in groups of four, with 12 h day/night cycle. The temperature of the animal lab was set at 21±2°C. The animals had free access to sufficient food and water. The protocols for use and care of animals were approved by Ethics Committee of Shahed University (Tehran, Iran) and were according to NIH guidelines.

2.2. Drugs

In this research study, morphine sulfate (Temad, Iran), methadone (Temad, Iran), haloperidol (Minoo, Iran), and naloxone (Tolid Daroo, Iran) were utilized. The injections were Intraperitoneal (IP) and each injection was 0.2 milliliters in volume.

2.3. Animal experiments

Ninety eight mice were divided into two groups of chronic and acute. Each group including 7 subgroups (n=7) 1- saline, 2- morphine (control),

3- methadone (10 mg/kg), 4- haloperidol (0.3 mg/kg) (18), 5- methadone+haloperidol (5 and 0.15 mg/kg, respectively), 6- methadone+haloperidol by 2 to 1 ratio (7 and 0.1 mg/kg, respectively), and 7- methadone+haloperidol by 1 to 2 ratio (3.5 and 0.2 mg/kg, respectively).

2.4. Drugs application methods

In order to create tolerance and dependence in mice in both chronic and acute groups, morphine was injected by increasing doses over a seven-day period, two times per day (08:00 A.M. and 04:00 P.M.) and on day 8 once (08:00 A.m.) according to the following schedule. Day one: 10, day two: 20, day three and four: 40, day five: 60, day six: 80, day seven: 100, and day eight: 100 mg/kg, IP (16) (The saline group received one dose of 100 mg/kg of morphine on day eight only).

In the chronic group, all medications in each subgroup were injected to the mice 30 minutes before all morphine doses over 8 days. In the acute group, the mice received one dose of the specific medication assigned to their subgroup only on day eight and 30 minutes before receiving the final morphine dose. It is notable that in both chronic and acute groups, morphine was injected to morphine (control) subgroup only according to the aforementioned schedule, and this subgroup received no further medications.

2.5. Pain tolerance test

In this experiment, the pain threshold of all of the mice on test day was assessed in two stages (30 minutes before drug injection and 30 minutes after morphine injection) by tail immersion test, in which one centimeter of the mouse's tail was put in water at a temperature of 56±0.5 degrees centigrade and the chronometer started immediately, pulling the tail out of water immediately after the animal showed a reflex, and stopping the chronometer. In this experiment, a cut-off time of 10 seconds was applied; if the animal showed no reflex for 10 seconds, the tail was pulled out from water. This test was repeated 3 minutes and the average was calculated. Afterwards, the average numbers were plugged into the following formula, to gain MPE: Maximal Possible Effect by percentage and the results were used for statistical analysis (17).

MPE%

$$\frac{\text{delay before morphine injection (sec)} - \text{delay after morphine injection (sec)}}{\text{delay before morphine injection (sec)} - \text{cut off time (sec)}} \times 100\%$$

2.6. Morphine withdrawal induction method

In order to induce morphine withdrawal syndrome to create symptoms and assess the levels of anxiety caused by withdrawal, 2 hours after the final morphine dose injection on test day (day 8), 5 mg/kg of naloxone was injected to each mouse. Each animal was separately placed in a transparent box of 20x20x30 cm afterwards, and behavioral symptoms were observed and recorded for 30 minutes. Mice show variable behaviors during the induction of the withdrawal syndrome. In this study, four quantitative behaviors, namely jumping times, rearing, front limbs licking, and diarrhea were assessed. Amongst these symptoms, jumping is of significant importance, to the extent that some researches have considered jumping alone to be sufficient for evaluating anxiety (18). Finally, 2 hours after naloxone injection, anxiety was evaluated.

2.7. Anxiety evaluation methods

In order to evaluate anxiety in the examined animals, elevated plus-maze and open field tests were used.

2.7.1 Elevated Plus-Maze test (EPM)

EPM test is an unconditional model for production and evaluation of anxiety and determination of the anxiogenic and anti-anxiety effects of medications. This test was first presented by Pellow et al. (19). The EPM device is a plus shaped wooden maze consisting of two open arms and two barred arms. The open arms were 40x10 centimeters in dimension and did not have walls. Barred arms are of the same size, but consist of 40 cm high side and end walls and are roofless. In the point of intersection of the four arms, a square of 10x10 centimeters is formed. The maze stands above ground on a column 50 centimeters high. The test is based on two principles: Investigative the instincts of the animal and their instinctive avoidance of open and bright spaces. In order to execute the test,

each mouse is separately placed in the central square facing an open arm, and moved freely in open and barred arms for 5 minutes. Times of entry to open and barred arms and the duration spent in open and barred arms were measured. Being in an arm for the animal is defined as a condition where all four limbs of the animal are inside the arm. The duration of presence in each arm is calculated on the same basis. Open arm entries percentage (OAE%) and open arm times percentage (OAT%) were used as standard anxiety assessment factors by the following formulas:

OAE% = entries to open arms divided by total entries to open and barred arms multiplied by 100.

OAT% = duration spent in open arms divided by total duration spent in open and barred arms multiplied by 100.

A significant increase in these parameters shows anxiety reduction.

2.7.2. Open Field test

The open field device is a cubic box (40x40x40 cm) made of Plexiglas. Its central area (20x20 cm) was separated using colored lines. The test, which is used to evaluate mouse anxiety, is based on the instinctive fear of being in the central area against tendency to discover and assess new environments. In order to execute the test, each mouse was individually placed in the field for 15 minutes, the primary situation of placement in the field being equal for all mice. The proportion of number of entries to the central area to the duration spent in the central area (seconds) was calculated afterwards. Anxious animals show a higher tendency to stay in the surrounding areas of the field. Higher proportions of entry to the central area to duration spent in central area show higher exploratory behavior and lower anxiety in the animal (20).

2.8. Statistical analysis

In the present study, the software SigmaStat version 3.5 (2006) was used for statistical analysis. All results were shown as Mean \pm Standard deviation. Statistical comparison between experimental groups was performed using one-way ANOVA and Tukey post-hoc tests and a level of $P < 0.05$ was chosen as significant. Non-parametric data analysis was executed using Kruskal-Wallis test and related post-test.

3. Results

3.1. Effects of methadone, haloperidol, and combination therapy on anxiety caused by morphine withdrawal in Plus-Maze and Open Field tests

As shown in Table 1 in elevated plus-maze test, open arm time percentage in treatment groups of haloperidol, methadone + haloperidol, and

methadone 1 + haloperidol 2 manifested with averages of 64.1 ± 9.71 , 51.68 ± 4.65 , and 54.63 ± 4.65 , respectively, which shows a significant increase as compared to the morphine group with an average of 14.04 ± 3 . Also, in this test, open arm entrance percentage in treatment groups of haloperidol, methadone + haloperidol, and methadone 1 + haloperidol 2 manifested with averages of 54.16 ± 3.37 , 53.61 ± 2.54 , and 52.61 ± 2.97 , respectively, which shows a significant increase as compared to the morphine group with an average of 38.03 ± 2.25 .

In the open field test, treatment groups of methadone, methadone 2 + haloperidol 1, and methadone 1 + haloperidol 2, with averages of 19.1 ± 4 , 29.2 ± 4.1 , and 22.2 ± 1.3 , respectively, as compared to the morphine group with an average of 7 ± 1.1 , had a significantly higher proportion of number of times of entrance to the central area to the time spent in this area.

Table 1. Effect of methadone, haloperidol, and chronic injection of the combination on morphine withdrawal-induced anxiety in Plus-Maze and Open Field tests

Open field test (%Number/duration)	Elevated plus maze test		Animal groups
	%Open arms entries	%Open arms time	
49.00 ± 5.00	58.04 ± 6.65	68.76 ± 9.74	Saline
7.00 ± 1.10	38.03 ± 2.25	14.04 ± 3.00	Morphine
$19.10 \pm 4.00^*$	50.00 ± 2.00	35.54 ± 7.04	Methadone
12.3 ± 2.00	$54.16 \pm 3.37^{**}$	$64.10 \pm 9.71^{***}$	Haloperidol
11.60 ± 4.30	$53.61 \pm 2.54^{**}$	$51.68 \pm 4.65^{***}$	Methadone+ Haloperidol
$29.20 \pm 4.10^{***}$	44.25 ± 3.07	36.31 ± 4.80	Methadone2+Haloperidol 1
$22.20 \pm 1.30^{**}$	$52.61 \pm 2.97^*$	$54.63 \pm 4.65^{***}$	Methadone1+Haloperidol 2

Results show Mean \pm SEM (n=7). *, **, and *** show significant difference relative to the morphine group ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

3.2. Effects of methadone, haloperidol, and combination therapy on anxiety caused by morphine withdrawal in Plus-Maze and Open Field tests

As shown in Table 2 in elevated plus-maze test, open arm spent time percentage in treatment groups of methadone and haloperidol with averages of 36.79 ± 4.03 and 41.23 ± 6.26 , and in

combination groups of methadone + haloperidol, methadone 2 + haloperidol 1, and methadone 1 + haloperidol 2 manifested with averages of 43.14 ± 5.85 , 30.52 ± 6.26 , and 37.53 ± 4.48 , respectively, which shows a significant increase compared to morphine group with an average of 14.04 ± 2.52 . Also, in this test, open arm entrance percentage in treatment groups of methadone and haloperidol with averages of 37.72 ± 3.34 and

41.33±3.7, and in combination groups of methadone + haloperidol, methadone 2 + haloperidol 1, and methadone 1 + haloperidol 2 manifested with averages of 39.66±4.8, 38.94±2.4, and 40.8±2.5, respectively, which shows a significant increase compared to morphine group with an average of 21.41±4.54.

In the open field test, treatment groups of methadone, methadone + haloperidol, and methadone 2 + haloperidol 1, with averages of 32.1±5.41, 39.2±2.5, and 46.5±4.36, respectively, compared to morphine group with average of 7.83±3.39, increased percentage of entrance times to central area to spent time in this area significantly.

Table 2. Effect of methadone, haloperidol, and combination acute injection on morphine withdrawal-induced anxiety in Plus-Maze and Open Field tests

Open field test (%Number/duration)	Elevated plus maze test		Animal groups
	%Open arms entries	%Open arms time	
67.00±17.00	43.88±3.09	68.76±9.74	Saline
7.83±3.39	21.41±4.54	14.04±2.52	Morphine
32.10±5.41*	37.72±3.34*	36.79±4.03*	Methadone
18.30±7.20	41.33±3.70**	41.23±6.26**	Haloperidol
39.20±2.50**	39.66±4.80*	43.14±5.85**	Methadone+ Haloperidol
46.50±4.36***	38.94±2.40*	30.52±6.26*	Methadone2+Haloperidol 1
23.10±2.17	40.80±2.50**	37.53±4.48*	Methadone1+Haloperidol 2

Results show Mean ± SEM (n=7). *, **, and *** show significant difference with morphine group (P<0.05, P<0.01, and P<0.001, respectively).

4. Discussion

In this study, the effects of methadone alone and co-drugs methadone and haloperidol therapy on the anxiety caused by morphine withdrawal in male mice were investigated. Results of this study showed that the anxious mice have lower percentage of open arm entries in the elevated plus-maze test and the time which spent in open arms. Also, the time waiting in central arena in the open field test were markedly decreased. These data indicate a high anxiety level in dependent mice during morphine withdrawal. Other studies have been reported that IP and intra amygdale (21, 22) application of morphine could yield anti-anxiety effects due to naloxone withdrawal in dependent rats. Also, some studies have shown that acute methadone injection causes a decrease in anxiety in both EPM and OF anxiety evaluation tests. Meany studies show the significant role of glutamate in promotion of anxiety, as activation of the NMDA glutamate receptors encouraged lab animals to nervousness (23, 24). On the other hand, it has been shown that systemic injection of MK801 and

phencyclidine, two non-competitive NMDA antagonists, in various anxiety evaluation tests such as plus-maze test, cause anti anxiety response induction (25, 26). It can be argued that based on existing reports, presumably methadone is capable of reduction of the anxiety caused by morphine withdrawal in dependent mice by blockage of NMDA glutamate receptors (27).

Results of this study on acute and chronic haloperidol prescription show that this medication is capable of increasing open arm entrance percentage in elevated plus-maze test significantly, and therefore, reducing the anxiety caused by morphine withdrawal in dependent mice. In some studies, the key role of NO/CGMP in anxiety neutralization has been demonstrated (8) and it has been shown that long term morphine administration results in further activation of CaMkII and therefore, higher production of nitric oxide. Based on this, we suspect that presumably haloperidol causes nitric oxide synthesis inhibition by inhibition of CaMkII and NOS enzyme, and hence, results in lower anxiety levels in mice. Along the same

lines with our results, some previous studies have proved nitric oxide synthetase enzyme to have anti-anxiety effects (28). On the other hand, it has been observed in neurochemical studies that following manifestation of naloxone-induced withdrawal syndrome symptoms in morphine addicted mice, glutamate and aspartate secretion highly increases in locus coeruleus nucleus and these stimulating amino acids have a significant role in higher noradrenaline secretion, which results in anxiety manifestation (29).

Regarding mentioned reports, perhaps haloperidol prevents higher glutamate release from the presynaptic neuron by inhibition of nitric oxide synthesis and thereby prevents higher noradrenaline secretion from noradrenergic neurons of locus coeruleus nucleus and reduces anxiety during morphine withdrawal. Dopamine is a neuronal mediator which has an important role in processes related to fear and anxiety. In some studies it has been shown that mesocorticolimbic dopaminergic pathway is involved in the effect of drugs on anxiety (30). Evidence shows that both D₁ and D₂ dopamine receptors are involved in anxiety mediation (31). However, D₂ dopamine receptors are of higher importance compared to D₁ receptors (32). According to some studies on anxiety, sulpiride anti psychotic medication prescription (D₂ receptor antagonist) results in decrease of anxiety and fear (14, 15).

On combinatory prescription of methadone and haloperidol, elevated plus-maze and open field anxiety tests results showed that acute prescription of these medications result in significant decrease in the anxiety caused by morphine withdrawal. Moreover, in chronic administration, all medications except methadone 2 + haloperidol 1 cause a significant decrease in anxiety. Hence, presumably in combinatory tests, medications reduce anxiety via a cumulative mechanism. According to what was mentioned above, it is expected that haloperidol prevented higher glutamate secretion from the presynaptic neuron and hence reduced noradrenaline secretion from locus coeruleus nucleus and therefore, decreased the anxiety level by having antagonist role to D₂ dopamine receptors and inhibition of CaMkII and nitric oxide synthetase enzyme activity. Also, methadone has had an effect as a NMDA receptor antagonist by blocking these receptors.

However, the results of this study showed that acute or chronic prescription of methadone and haloperidol drug combination causes reduction in the anxiety caused by morphine withdrawal. Since methadone is known as a regular medication for drug addicts and also regarding the anti-psychotic effects of haloperidol as an effective medication in treatment of mental disorders, it is suggested that perhaps a combination of the medications mentioned above could be used as a higher effective way of prevention and treatment of anxiety due to narcotic disruption. Therefore, supplementary research is necessary in further studies.

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