Co-administration of calcium gluconate and magnesium acetate effectively blocks the signs of morphine withdrawal in mice

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Abstract. The present study was conducted to investigate the effect of oral administration of calcium gluconate and magnesium acetate on morphine withdrawal syndrome. Mice were rendered dependent on morphine by subcutaneous injection of increasing doses of morphine. Mice were observed for 30 minutes for the withdrawal signs (jumping or standing events, diarrhea, piloerection, tremor and ptosis). Separate oral administration of magnesium (50, 75 and 100 mg/kg) and calcium (500, 750 and 1,000 mg/kg) significantly decreased the jumping, without affecting standing in animals withdrawn from morphine. Co-administration of magnesium (at a fixed dose of 100 mg/kg) and calcium (at a range of doses from 250 to 1,000 mg/kg) resulted in a significant reduction in jumping and standing events (P < 0.05). In a similar fashion, the qualitative signs of withdrawal were also reduced when the above combination of calcium and magnesium was administered. Co-administration of calcium/magnesium at 500/50, 750/75 and 1,000/100 mg/kg significantly reduced the number of jumps in morphine-dependent animals without affecting the number of standing events. This study demonstrates the potential activity of the co-administration calcium and magnesium in preventing the signs associated with morphine withdrawal syndrome.

Key words: calcium, magnesium, morphine, withdrawal, dependence

Introduction

Opioid analgesics such as morphine are widely used in treatment of acute and chronic pain, but their clinical use is greatly limited by the development of tolerance and dependence produced by prolonged administration [19]. Considering the widespread use of morphine for the management of severe pain, the reluctance to consider chronic administration has broad clinical implications [13]. In order to develop medications that can prevent the development of dependence or to reverse existing dependence, it is essential to identify the underlying mechanisms of physical dependence. Despite decades of research, our understanding of the mechanisms underlying the physical dependence associated with opiates, and their withdrawal is still very limited, and several mechanisms may operate in these processes [20, 25, 26]. Based on the interaction between opiate and non-opiate receptor systems in the CNS, several groups of drugs have been tested for their effects on the development of tolerance and physical dependency [26]. None of these drugs, however, has proven to be completely effective and none is without drawbacks.
A large body of data accumulated from different laboratories over the past decade suggests that the N-methyl-d-aspartate (NMDA) receptor and its second messenger system play a critical role in the development of morphine tolerance and dependence [13, 16, 25]. Behavioural studies have shown that administration of non-competitive or competitive NMDA receptor antagonists can attenuate the expression of the withdrawal syndrome in animals when administered immediately before naloxone-precipitated morphine withdrawal [20]. Magnesium is a non-competitive NMDA antagonist that modulates receptor activation by blocking the NMDA receptor channel [21]. Many studies have shown the attenuation of morphine tolerance and dependence by magnesium [3, 27]. The other mechanism that has been well studied and that is thought to play a major role in the development of tolerance to and dependence on opiates is that of calcium channels [15]. Chronic administration of opiates to laboratory animals has been shown to produce an increase in calcium uptake into various brain preparations [12, 24]. Consistent with these results, calcium channel blockers have been shown to decrease the magnitude of tolerance and reduce in vivo opioid agonist-induced downregulation of μ-opioid receptors [6, 7, 18]. The number of dihydropyrdine-sensitive binding sites in the CNS, thought to represent voltage-sensitive calcium channels, was increased in rats showing the signs of morphine withdrawal [2, 23].

Calcium/magnesium soft gels (CalMag) are pH-balanced sources of calcium and magnesium that are available as a nutritional supplement. In addition to calcium (1,000 mg) and magnesium (500 mg), each capsule of CalMag also contains vitamin D, zinc, lecithin, soybean oil, gelatin, glycerin and water. These capsules are primarily used to aid in the development and maintenance of bones. Acute administration of magnesium and calcium in the form of a soft gel capsule was shown to decrease significantly the signs of morphine withdrawal syndrome [22]. In a recent study, we showed that the main ingredients of these capsules i.e. calcium and magnesium when given chronically via the intraperitoneal route also blocked the development of tolerance and dependence. Since magnesium and calcium can be absorbed orally, the aim of this study was to evaluate the effects of these two elements, alone or in combination, in naloxone-treated, morphine-withdrawn mice. It may be possible to advance a step further towards testing the drug in human subjects by introducing it into the daily diet of morphine-addicted patients.

**Methods and materials**

**Animals**

Male NMRI mice (Pasteur institute, Tehran, Iran) weighing 25-30 g were housed in cages of six, with controlled room temperature and a 12 h light-dark cycle. Food and water were available *ad libitum*. Tests were performed only after the mice had acclimated to the above environment for at least seven days. All experiments were conducted between 09:00 and 13:00 every day to avoid any temporal factor (e.g., circadian rhythm). Each animal was used for only one experimental condition. Animals were housed and used in accordance with the guidelines of the committee on the care and use of laboratory animal resources, of the Isfahan University of Medical Sciences.

**Drugs and method of administration**

Morphine sulfate (TEMAD, Iran), was dissolved in normal saline (0.9%) and injected via the s.c. route. Naloxone hydrochloride was purchased as an ampoule (0.4 mg/mL, TEMAD, Iran), and injected i.p. without diluting to induce morphine-withdrawal syndrome. Calcium gluconate and/or magnesium acetate were dissolved in saline and administered to animals via a feeding needle. All the drugs were given in a constant volume of 10 mL/kg body weight, and the control animals received the equivalent volume of vehicle.

**Morphine-withdrawal syndrome**

Groups of 6-9 mice were chosen randomly for each type of treatment. Morphine was injected twice daily at 08:00 and 18:00 for five days following an escalating dose schedule, i.e. 1st day (30 and 30 mg/kg at 08:00 and 18:00, respectively), 2nd day (45 and 45 mg/kg), 3rd day (60 and 60 mg/kg), 4th day (90 and 90 mg/kg) and 5th day (90 mg/kg at 08:00 only). Calcium and/or magnesium and vehicle were given orally, one h prior to the naloxone injections.
Figure 1. The effect of oral administration of separate doses of calcium and magnesium on (A) number of stands and (B) number of jumps induced by naloxone in morphine-dependent mice. Morphine was given in increasing doses (from 15 to 90 mg/kg) over a period of five days as described in “Methods and materials”. Magnesium and calcium were injected one h after the last dose of morphine (one h before the naloxone injection). Withdrawal signs were precipitated by naloxone (5 mg/kg) and observed for 30 min. Results are the mean (± SEM) from groups of 6-9 mice. *P<0.05 versus saline control using one-way ANOVA followed by a Newman-Keuls post hoc comparison.

Withdrawal signs were precipitated by injecting naloxone (5 mg/kg), two h after the final injection of morphine. Immediately after a naloxone challenge, mice were placed individually in an observation box and withdrawal signs were evaluated over a 20 min period by counting the number of times the animals jumped and stood. A selected number of qualitative and quantitative signs were rated in accordance with the Gellert and Holtzman rating scale [9]. These are signs that are easier to evaluate at an early phase of drug screening, and normally consists of graded and checked signs. Graded signs such as piloerection, rapid breathing, eye ptosis and diarrhea, were assigned a weighting factor from 1 to 4 which was based on the frequency of their appearance.

Statistical analyses
Quantitative data were assessed using Student’s t-test and one-way analysis of variance (ANOVA) with a post hoc Newman-Keuls test and expressed as mean ± S.E.M. Qualitative scores were analyzed using the Mann-Whitney test and were expressed as median ± interquartile ranges. In all comparisons, P<0.05 was considered significant.
Results

Normal saline (control group), calcium and/or magnesium were administered orally at different doses, one h before i.p. injection of naloxone to the mice that had been receiving morphine chronically. In these morphine-dependent mice, the naloxone injection precipitated the standard behavioral signs of withdrawal such as jumping, standing, diarrhea, and urination. In the drug-naive group however, the injection of naloxone did not trigger such behavioral changes.

Effects of separate doses of calcium or magnesium

To find the minimum effective dose, calcium or magnesium were administered orally in varying doses. As shown in figure 1, oral administration of calcium at 500, 750 and 1,000 mg/kg significantly decreased the number of jumps without affecting the number of standing events in a withdrawal-like syndrome that was precipitated by injection of naloxone (p<0.05). A similar pattern of results was also observed when magnesium at 50, 75 and 100 mg/kg was administered. At doses lower than 500 mg/kg, calcium did not produce any significant effect on either the number of standing or jumping events, whereas a concentration higher than 1,000 (e.g., 1,500 mg/kg) produced profound behavioral effects such as abdominal constriction (data not shown). Magnesium at concentrations higher than 100 mg/kg were not used as it produced some behavioral changes in the animals (data are not shown). As far as the qualitative signs of withdrawal were concerned, magnesium at higher doses significantly attenuated the withdrawal signs such as diarrhea, piloerection, and tremor although with little effect on ptosis (table 1). Calcium on the other hand, was only effective in blocking some of these signs at the high dose of 1,000 mg/kg.

Co-administration of magnesium and calcium

Fixed dose of magnesium with a range of doses of calcium
Calcium and magnesium were co-administered in the next set of experiments, keeping the dose of magnesium fixed, while varying the dose of calcium. Administration of magnesium (at a fixed dose of 100 mg/kg) and calcium (at a range of doses from 250 to 1,000 mg/kg) resulted in a significant reduction in the number of jumping and standing events in morphine-withdrawn mice (P<0.05, figures 2A and 2B). In a similar fashion, the qualitative signs of withdrawal were also reduced when the above combination of calcium and magnesium was administered (table 2).

Mixed doses of calcium and magnesium
Three doses of calcium and magnesium combined were tested next. Co-administration of calcium/magnesium at 500/50, 750/75 and 1,000/100 mg/kg significantly reduced the number of jumps without affecting the number of standing events in morphine-dependent animals (P<0.05, figure 3).

Table 1. The effect of oral administration of various doses of calcium and magnesium on naloxone-precipitated withdrawal signs in morphine-dependent mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median behavioural scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diarrhea</td>
</tr>
<tr>
<td>Control</td>
<td>4 (3-4)</td>
</tr>
<tr>
<td>Magnesium (50 mg/kg)</td>
<td>1 (1-2)*</td>
</tr>
<tr>
<td>Magnesium (75 mg/kg)</td>
<td>2 (1-2)*</td>
</tr>
<tr>
<td>Magnesium (100 mg/kg)</td>
<td>1.5 (1-2)*</td>
</tr>
<tr>
<td>Calcium (500 mg/kg)</td>
<td>3.5 (3-4)</td>
</tr>
<tr>
<td>Calcium (750 mg/kg)</td>
<td>3 (2-3)</td>
</tr>
<tr>
<td>Calcium (1,000 mg/kg)</td>
<td>2 (1-2)*</td>
</tr>
</tbody>
</table>

Morphine was given in increasing doses (from 15 to 90 mg/kg) over a period of five days as described in “Methods and materials”. Magnesium and calcium were injected one h after the last dose of morphine (one h before the naloxone injection). The withdrawal was precipitated by naloxone (5 mg/kg); withdrawal signs were observed for 30 min. *P<0.05 versus saline control using one-way ANOVA followed by a Mann-Whitney post hoc comparison. The results are the median scores for withdrawal signs (± interquartile ranges in parenthesis)
Figure 2. The effect of oral, co-administration of a fix dose of magnesium and varying doses of calcium (A) number of stands and (B) number of jumps induced by naloxone in morphine-dependent mice. Morphine was given in increasing doses (from 15 to 90 mg/kg) over a period of five days as described in “Methods and materials”. Magnesium and calcium were injected one h after the last dose of morphine (one h before the naloxone injection). Withdrawal signs were precipitated by naloxone (5 mg/kg) and observed for 30 min. Results are the mean (± SEM) from groups of 6-9 mice. *P<0.05 versus saline control using one-way ANOVA followed by a Newman-Keuls post hoc comparison.

The combination of calcium/magnesium at 1,000/100 was the most effective dose in blocking the qualitative signs of withdrawal (table 3). Other combined doses of these two elements attenuated some signs, without affecting others (table 3).

Discussion

Previous studies have shown that a combination of calcium and magnesium given via the i.p. route attenuates the signs of morphine withdrawal [14, 22]. The current study was designed to evaluate the effects of three, orally administered, combined doses of calcium and magnesium in morphine-dependent mice. The doses of calcium and magnesium were based on the original, soft gel capsule. To induce morphine dependence, different programs have been used and a wide range of behaviors evaluated [4]. In this study, morphine, in increasing dose over a four-day period followed by naloxone, produced a full-blown withdrawal syndrome. This program was used to evaluate the ability of magnesium and/or calcium to inhibit...
Table 2. The effect of oral, co-administration of various doses of calcium and a fixed dose of magnesium on naloxone-precipitated withdrawal signs in morphine-dependent mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diarrhea</th>
<th>Piloerection</th>
<th>Tremor</th>
<th>Eye ptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4 (4-4)</td>
<td>3 (3-4)</td>
<td>4 (3-4)</td>
<td>4 (3-4)</td>
</tr>
<tr>
<td>Ca/Mg (250/100 mg/kg)</td>
<td>1.5 (1-2)*</td>
<td>2 (2-3)*</td>
<td>1.5 (1-2)*</td>
<td>1 (1-2)*</td>
</tr>
<tr>
<td>Ca/Mg (500/100 mg/kg)</td>
<td>1.5 (1-2)*</td>
<td>2 (2-3)*</td>
<td>1.5 (1-2)*</td>
<td>1 (1-2)*</td>
</tr>
<tr>
<td>Ca/Mg (750/100 mg/kg)</td>
<td>1 (1-2)*</td>
<td>2 (1-2)*</td>
<td>1.5 (1-2)*</td>
<td>1 (1-2)*</td>
</tr>
</tbody>
</table>

Morphine was given in increasing doses (from 15 to 90 mg/kg) over a period of five days as described in “Methods and materials”. Magnesium and calcium were injected one h after the last dose of morphine (one h before the naloxone injection). The withdrawal was precipitated by naloxone (5 mg/kg); withdrawal signs were observed for 30 min. *P < 0.05 versus saline control using one-way ANOVA followed by a Mann-Whitney post hoc comparison. The results are the median scores for withdrawal signs (± interquartile ranges in parenthesis).

withdrawal signs such as jumping, standing, diarrhoea, piloerection, tremor and ptosis.

Based on our pilot data and number of other related studies, three doses of calcium and magnesium were selected for this work. The results of the first series of experiments showed that oral administration of separate doses of calcium and magnesium significantly suppressed the number of jumps without affecting number of standing events or other quantitative withdrawal signs. This is in contrast to the previous finding where acute i.p. administrations of calcium (50 mg/kg) or magnesium (25 mg/kg) were shown to be ineffective in reducing the number of jumps [22]. The doses in this study however, were higher than those used when these two elements were given via the i.p. route. Higher doses are normally required when the drugs are given via the oral route to compensate for incomplete absorption.

Combinations of calcium and magnesium were proven to be more effective than their separate oral administration in alleviating symptoms of withdrawal. Jumping was most effectively blocked by the combination of these two elements, although other qualitative withdrawal symptoms were also affected. Jumping is one the most common measures that is used to quantify the intensity of morphine withdrawal. This parameter has been used on many occasions as the only sign in the evaluation of opiate dependence in mice [4]. Most of the combined doses of calcium and magnesium that were tested in this study provided approximately similar potency in blocking the quantitative signs, signifying the importance of the two elements being present together.

Although the use of magnesium is widespread and effective, its underlying mechanism of action is not known. Several possible mechanisms of action have been proposed, including it action as a unique calcium antagonist as it can act on most types of calcium channels in vascular smooth muscle [1]. Chronic administration of morphine and other opioid agonists has been shown by several groups to produce an increase in the number of dihydropyridine binding sites in membranes prepared from dissected brain regions [15]. This increase in calcium channel numbers could be an important adaptation mechanism for countering the decrease in intraneuronal calcium caused by morphine. When morphine treatment is stopped, these channels remain, at least temporarily, unopposed causing massive excitation in the CNS. Magnesium and indeed other calcium channel blockers could prevent the withdrawal syndrome by limiting the inflow of calcium [18]. The peripheral and cerebral vasodilatory effects of magnesium are also thought to be, at least in part, mediated by a blockade of calcium channels [8]. One major effect of decreased intracellular calcium would be inactivation of calmodulin-dependent myosin light chain kinase activity and decreased contraction, causing arterial relaxation that may subsequently lower peripheral and cerebrovascular resistance, relieve vasospasm, and decrease arterial blood pressure [1].

The other possible mechanism for the protective effect of magnesium may be related to its role as an N-methyl-D-aspartate (NMDA) receptor antagonist. Hyperactivity that is seen during abstinence from morphine is thought to be mediated, at least in part, by stimulation of glutamate receptors, such as the NMDA receptors that are up-regulated during chronic morphine treatment. Magnesium as an NMDA receptor antagonist blocks the...
Figure 3. The effect of oral, co-administration of doses of both magnesium and calcium (A) number of stands and (B) number of jumps induced by naloxone in morphine-dependent mice. Morphine was given in increasing doses (from 15 to 90 mg/kg) over a period of five days as described in “Methods and materials”. Magnesium and calcium were injected one h after the last dose of morphine (one h before the naloxone injection). Withdrawal signs were precipitated by naloxone (5 mg/kg) and observed for 30 min. Results are the mean (± SEM) from groups of 6-9 mice. *P<0.05 versus saline control using one-way ANOVA followed by a Newman-Keuls post hoc comparison.

calcium influx and attenuates the development of morphine tolerance and dependence [3, 17, 27]. Systemic magnesium treatment results in a resistance to both electrically stimulated [10] and NMDA-induced hippocampal seizures [5]. Furthermore, systemic treatment with magnesium causes a significant reduction in the NMDA receptor-binding capacity in the brain [11]. Some studies have shown that drug abuse depletes magnesium from the body. The benefits of magnesium in reducing opiate dependence may be due to the replenishing of a deficiency of this mineral. The amount of magnesium used in this study was moderate; however, higher amounts may be necessary for individuals who are more deficient.

Magnesium ions must cross the BBB to elicit central effect. It has been demonstrated in animals that MgSO₄ can cross the intact BBB and enter the central nervous system, and this correlates with the level of serum hypermagnesemia [11]. The entry of magnesium into the brain could increase in line with withdrawal hyperexcitability. Evidence shows that seizure activity increases the movement of magnesium into the brain [10].
Table 3. The effect of oral, co-administration of various doses of calcium and magnesium on naloxone-precipitated withdrawal signs in morphine-dependent mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diarrhea (median)</th>
<th>Piloerection (median)</th>
<th>Tremor (median)</th>
<th>Eye ptosis (median)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4 (3-4)</td>
<td>3 (3-3)</td>
<td>4 (4-4)</td>
<td>3.5 (3-4)</td>
</tr>
<tr>
<td>Ca/Mg (500/50 mg/kg)</td>
<td>4 (3-4)</td>
<td>2 (2-3)*</td>
<td>1 (1-2)*</td>
<td>3 (3-4)</td>
</tr>
<tr>
<td>Ca/Mg (750/75 mg/kg)</td>
<td>1.5 (1-2)*</td>
<td>4 (3-4)</td>
<td>2.5 (2-4)*</td>
<td>2 (2-2)*</td>
</tr>
<tr>
<td>Ca/Mg (1,000/100 mg/kg)</td>
<td>1 (1-1)*</td>
<td>1 (1-2)*</td>
<td>1 (1-1)*</td>
<td>1 (1-2)*</td>
</tr>
</tbody>
</table>

Morphine was given in increasing doses (from 15 to 90 mg/kg) over a period of five days as described in “Methods and materials”. Magnesium and calcium were injected one h after the last dose of morphine (one h before the naloxone injection). The withdrawal was precipitated by naloxone (5 mg/kg); withdrawal signs were observed for 30 min. *P<0.05 versus saline control using one-way ANOVA followed by a Mann-Whitney post hoc comparison. The results are the median scores for withdrawal signs (± interquartile ranges in parenthesis).

The protective effect of calcium does not comply with the general regulation, since, as described earlier, inhibition of calcium flow by calcium antagonists blocks the development of the signs of morphine withdrawal [15]. Therefore, calcium is thought to potentiate the development of morphine dependence and not attenuate it. The effects of calcium on the development of tolerance and dependence on morphine have always been contradictory. What probably happens in the current type of setting is that calcium, by increasing magnesium absorption, potentiates its CNS effects. Calcium is one of the major elements that influence magnesium absorption in the body.

This study demonstrates the potential effect of the co-administration of calcium and magnesium in alleviating the symptoms of morphine withdrawal in mice. Addition of a formulated compound, containing optimum amounts of calcium and magnesium, to the diet of addicts could contribute to an effective detoxification program.

Disclosure

This work was supported by the research department of the Isfahan University of Medical Sciences.

References


