Experimental paper

Protective effects of calcium-magnesium soft gels in morphine tolerant and dependent mice

M. Rabbani*, H.M. Sadeghi, S. Gudarzi

Department of Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Correspondence: Dr. M. Rabbani, Department of Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran. Tel.: (+00 98) 311 7922646. Fax: (+00 98) 311 6680011. <rabbani@pharm.mui.ac.ir>

Abstract. The present study was aimed at evaluating the acute effects of Calcium-Magnesium soft gels (CalMag) in morphine tolerant and dependent mice. Mice were rendered tolerant and dependent on morphine by subcutaneous injection of morphine over a fixed time period. Withdrawal signs were precipitated by injecting naloxone 2 h after the final injection of morphine. The tail-pinch assay was used to investigate the effects of various compounds on the development and reversal of morphine tolerance. Acute injection of CalMag (containing 50 mg/kg calcium and 25 mg/kg magnesium) significantly reduced the number of jumps, stands and fast breathing in morphine dependent mice. Co-administration of calcium (50 mg/kg) and magnesium (25 mg/kg) was also effective in preventing the development of morphine tolerance and dependence. Administration of calcium (up to 50 mg/kg) alone did not significantly block the development of tolerance and dependence. The mean latency to pain was significantly increased in animals pretreated with CalMag (containing 50 mg/kg calcium and 25 mg/kg magnesium). The mixture of calcium and magnesium at specific concentrations seem to be critical for preventing the development of morphine tolerance and dependence.

Keywords: calcium magnesium softgels, morphine, withdrawal, tolerance

Opiate drugs such as morphine are widely used in the clinical management of pain. Their clinical usefulness, however, is limited by tolerance and dependence. A detailed understanding of molecular mechanisms of morphine tolerance and physical dependence is considered to be essential for treating and prevention of this phenomenon. Large numbers of drugs with different pharmacological mechanisms have been tested for their effects on morphine tolerance and physical dependence [1-7]. None of these drugs, however, have proven to be completely effective and without drawbacks.

Calcium magnesium softgels (CalMag) are pH-balanced, multiple-source calcium and magnesium that are available as nutritional supplements. In addition to calcium (1,000 mg) and magnesium (500 mg), each capsule of CalMag also contains vitamin D, zinc, lecithin, soy bean oil, gelatin, glycerine and water. These capsules are primarily used to aid in the development and maintenance of bones. Recently, some addiction treatment centres have used CalMag capsules to treat morphine addicts. Although there is no scientific finding to support this effect of morphine, magnesium alone has been shown to have much involvement in central nervous system (CNS) function. In this study, therefore, we aimed at evaluating the effects of CalMag capsules in morphine tolerant and dependent mice. Further-
more and in order to eliminate the effects of other components of the CalMag capsules, we compared the effects CalMag with that of calcium and/or magnesium, separately.

**Methods**

**Animals**

Male Syrian mice (Pasture, Tehran) weighing 25-30 g were housed in a cage with controlled room temperature 22-25 degrees. Food and water were available ad libitum. Tests were performed only after the mice had been acclimatised to the above environment for at least 7 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 and 6 animals in a group. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals at Isfahan University of Medical Sciences (2002).

**Drugs and methods of administration**

Morphine sulfate (TEMAD, Iran), and naloxone hydrochloride (TEMAD, Iran) were dissolved in distilled water. The content of one capsule of CalMag soft gel (Bluebonnet Nutrition Corporation, USA) was carefully suspended in distilled water containing 0.5% Tween 80. The drug solution was brought up to the final concentration while warming to 40°C with vigorous shaking. Calcium sulphate (CaSO$_4$) and magnesium chloride (MgCl$_2$) were prepared in distilled water and used separately or mixed in appropriate amounts to give the final required concentrations. Two different doses of CalMag were used in our study. The first one was set to give final calcium and magnesium doses of 50 mg/kg and 25 mg/kg, respectively (CalMag 50/25). The second dose was set to give final calcium and magnesium doses of 25 mg/kg and 12.5 mg/kg magnesium (CalMag 25/12.5). Morphine was administered subcutaneously (s.c.) while naloxone and CalMag were given intraperitoneally (i.p.) in a constant volume of 10 mL/kg body weights. The control animals received the equivalent volume of vehicle.

**Morphine withdrawal syndrome**

Morphine was injected s.c. twice daily at 08:00 and 18:00 for 5 days according to an escalating dose schedule. The morphine dose was increased progressively from 30 to 90 mg/kg over a period of 5 days, i.e. 1$^{st}$ day (30 and 30 mg/kg at 08:00 and 18:00, respectively), 2$^{nd}$ day (45 and 45), 3$^{rd}$ day (60 and 60), 4$^{th}$ day (90 and 90) and 5$^{th}$ day (90 mg/kg at 08:00 only). The control mice received s.c. vehicle injections. In saline-treated mice, saline was administered twice daily for 5 days according to the same injection schedule.

Withdrawal signs were precipitated by injecting naloxone (5 mg/kg, i.p.) 2 h after the final injection of morphine. CalMag, calcium and/or magnesium and vehicle were injected (i.p.) 30 min after the final dose of morphine and 90 min prior to naloxone administration. Immediately after a naloxone challenge, mice were individually placed in an observation box and were observed for 15 min for the occurrence of withdrawal-related behaviors. The signs of withdrawal, were evaluated either by scoring the intensity of the signs from 0 to 4 points (hair raising, sniffing, fast breathing and diarrhea) or by counting the number of events (jumping and standing).

**Tolerance paradigms**

A 4-day cumulative dosing regimen was used for the induction of morphine tolerance. The treatment schedule consisted of twice daily s.c. doses of morphine given at 20 mg/kg (08:00 and 18:00). Tolerance was assessed on the 5th day based on loss of the antinociceptive effects of morphine (20 mg/kg, s.c.), using the modified Haffner’s methods, the tail-pinch test [8].

For the tail-pinch assay, a flattened clip (approximately 10 mm wide) was placed approximately 1 inch from the base of a mouse tail, and only the mice that responded to the clip placement by turning or biting at the clip within 15 s were used in this test. Drug-naive mice responded to this pressure by immediately vocalizing and biting at the clip. The clip was never applied to the mouse’s tail for longer than 15 s. An animal that failed to respond before 15 s (cut-off time) was removed from the apparatus and assigned a latency of 15 s. CalMag, calcium and/or magnesium and vehicle were administered i.p. 30 min after the final morphine injection. The tail-pinch response was determined 2 h after the final morphine injection.

**Statistical analyses**

Quantitative data were assessed using one-way analysis of variance (ANOVA), with post-hoc Newman-Keuls test and expressed as mean ± SEM. Qualitative scores were analyzed with one-way ANOVA followed by the Dunn’s test for post-hoc comparisons and expressed as median ± interquartile ranges. In all comparisons, p < 0.05 was considered significant.
Results

Morphine dependence and naloxone challenge
In mice injected with morphine for 5 days, naloxone administration precipitated the standard behavioral signs of withdrawal (jumping, standing, sniffing, fast breathing and diarrhea). In saline injected control groups, however, the injection of naloxone did not trigger behavioral changes.

Effect of CalMag on morphine withdrawal signs
Different doses of the CalMag capsules were first tested in control animals. As the concentration of calcium and magnesium are most crucial to adjust in our preparations, the capsules were diluted in a way to give a final calcium and magnesium dose of 50 mg/kg and 25 mg/kg, respectively (CalMag 50/25). This was the maximum tolerable concentration of CalMag in mice, doses above this caused severe overt reaction (e.g. tremor, hyperventilation). CalMag 25/12.5 was a solution of CalMag that gave final calcium and magnesium doses of 25 mg/kg and 12.5 mg/kg, respectively.

As is illustrated in figure 1 and table 1, CalMag 50/25 significantly reduced the number of jumping and other qualitative signs of morphine withdrawal (p < 0.05). The number of stands, however, was only significantly reduced by CalMag 50/25 and not 25/12.5 (figure 2). CalMag at doses lower than 25/12.5, did not alter any of the measured behavioural signs (data not shown).

In the next study, we compared the effect of a separate solution containing similar concentration

![Figure 1](image-url)

**Figure 1.** Effects of calcium magnesium softgel (CalMag) and calcium and/or magnesium on the number of jumps induced by naloxone after the cessation of chronic morphine treatment. Morphine was given in increasing dose (from 15 to 90 mg/kg) over a period of 5 days as described in methods. CalMag, calcium and/or magnesium and vehicle were injected in a single dose, 30 min after the final dose of morphine and 90 min prior to naloxone administration. The withdrawal was induced by naloxone (5 mg/kg), and recorded for 15 minutes. Results are the mean jumping (± S.E.M.) from group of 6 mice. * p < 0.05 for comparison between morphine plus test compounds with morphine plus saline group.
Figure 2. Effects of calcium magnesium softgel (CalMag) and calcium and/or magnesium on the number of stands induced by naloxone after the cessation of chronic morphine treatment. Morphine was given in increasing doses (from 15 to 90 mg/kg) over a period of 5 days as described in methods. Calcium magnesium softgels, calcium and/or magnesium and vehicle were injected in a single dose, 30 min after the final dose of morphine and 90 min prior to naloxone administration. The withdrawal was precipitated by naloxone (5 mg/kg), and the signs were observed for 15 min. Results are the mean jumping (± S.E.M.) from group of 6 mice. * p < 0.05 for comparison between morphine plus test compounds with morphine plus saline group.

Table 1. The effects of Calcium magnesium softgel and calcium and/or magnesium (CalMag) on naloxone-precipitated withdrawal signs in morphine dependent mice.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fast breathing</th>
<th>Freezing</th>
<th>Diarrhea</th>
<th>Ptose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>4 (3-4)</td>
<td>3 (2-3)</td>
<td>3 (1-3)</td>
<td>3 (1-3)</td>
</tr>
<tr>
<td>CalMag (50/25)</td>
<td>2* (2-3)</td>
<td>1.5* (1-2)</td>
<td>1* (1-2)</td>
<td>2* (1-3)</td>
</tr>
<tr>
<td>CalMag (25/12.5)</td>
<td>3* (3-4)</td>
<td>1.5* (1-2)</td>
<td>1* (1-2)</td>
<td>2* (1-3)</td>
</tr>
<tr>
<td>Ca (50 mg/kg)</td>
<td>3.75 (2-4)</td>
<td>2 (1-2)</td>
<td>2.5 (1-3)</td>
<td>3 (2-4)</td>
</tr>
<tr>
<td>Mg (25 mg/kg)</td>
<td>2* (2-2)</td>
<td>2.5* (2-3)</td>
<td>1* (1-3)</td>
<td>2* (2-3)</td>
</tr>
<tr>
<td>Ca (50 mg/kg) + Mg (25 mg/kg)</td>
<td>2* (2-3)</td>
<td>1.5* (1-3)</td>
<td>1* (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 5 days as described in methods. CalMag, calcium and/or magnesium and vehicle were injected in a single dose, 30 min after the final dose of morphine and 90 min prior to naloxone administration. The withdrawal was precipitated by naloxone (5 mg/kg), and the signs were observed for 15 min. The results are the median scores for withdrawal signs (+ interquartile ranges in parenthesis, n = 6). * p < 0.05 for comparison between morphine plus test compounds with morphine plus saline group.
of calcium (50 mg/kg) and magnesium (25 mg/kg) as in the CalMag capsules on morphine withdrawal signs. Co-administration of calcium (50 mg/kg) and magnesium (25 mg/kg) was also effective in significantly reducing the number of jumps and stand in morphine dependent mice (figures 1 and 2, p < 0.05). Administration of calcium alone up to 50 mg/kg did not significantly alter any of the measured morphine withdrawal signs (figures 1 and 2, p < 0.05). Administration of calcium alone up to 50 mg/kg did not significantly alter any of the measured morphine withdrawal signs (figures 1, 2, and table 1). In contrast, a magnesium injection at 25 mg/kg worsened the withdrawal syndromes by increasing the number of jumps and therefore the higher concentration was not tested. In saline injected control groups, the injection CalMag 50/25, calcium (lower than 50 mg/kg) and/or magnesium (lower than 50 mg/kg) did not affect their normal behaviour.

Effects of CalMag on development of morphine tolerance

The tail-pinch test was used to investigate the effects of acute administration of the CalMag, and calcium and/or magnesium on the development and reversal of morphine tolerance. Based upon the results obtained in the tail-pinch test, a single dose of 20 mg/kg morphine produced antinociceptive effects in naive mice that was observed for the 15 s of cut-off period and lasted for about 2 hours. The mean response latency to nociceptive stimuli was gradually shortened from the first dose (15 s) of morphine to 1.17 ± 0.17 s (n = 12) on day 5.

Figure 3 shows the mean response latency to nociceptive stimuli in morphine tolerant mice pretreated with CalMag, calcium and/or magnesium or vehicle.
Pretreatment with CalMag 50/25 or 25/12.5 significantly inhibited the development of morphine tolerance. The mean latency responses to pain, in mice pretreated with 50/25 and 25/12.5 of CalMag were 14.2 and 12.8 s, respectively. Similarly, pretreatment with magnesium (25 mg/kg) or the mixture of calcium (50 mg/kg) and magnesium (25 mg/kg) significantly inhibited the development of tolerance to morphine. Calcium, up to the dose of 50 mg/kg, however, did not reverse the development of tolerance to morphine (figure 3).

Discussion

Calcium magnesium softgels are multiple-sources of calcium and magnesium that are primarily used to aid in the development and maintenance of bones. Recently, some addiction treatment centers in Iran and other countries have used the CalMag capsules to treat drug addicts. The aim of the present study was to investigate the effects of the CalMag capsules on morphine tolerant and dependent mice.

The schedule of chronic morphine treatment produced tolerance and physical dependence which was exhibited by various qualitative (fast breathing, freezing, diarrhea and ptose) and quantitative (jumping and standing) signs, after injection of naloxone. In the naloxone-precipitated withdrawal study, a single injection of CalMag (50/25) 1 h after the last morphine injection (1 h before naloxone) significantly reduce most signs of withdrawal. In the tail pinch assay, CalMag (50/25) also prevented the development of tolerance to morphine. In order to eliminate the influence of other ingredients of the CalMag capsules (vitamin D, zinc, lecithin, soy bean oil, gelatin, glycerine and water), we tested the effects of calcium and/or magnesium separately. In these studies, co-administration of calcium and magnesium (similar in concentration to that of the capsules) provided the best protective effects against morphine tolerance and dependence. Further experiments with the administration of calcium or magnesium alone pointed to the importance of magnesium in these drugs mixtures.

The mechanisms underlying the inhibitor effects of magnesium on opiate physical dependence and tolerance are not clear. Magnesium could exert its effects via at least two different pathways in the brain. One possibility is that chronic morphine treatment up-regulates the calcium channels and withdrawal from opiates results in compensatory hyperactivity that produces an increase in brain calcium concentration [3]. Magnesium has been referred to as a physiological Ca channel blocker, in the sense that processes activated or stimulated by calcium ions may be deactivated or inhibited by magnesium ions [9, 10]. By blocking calcium channels, magnesium could inhibit the influx of calcium and therefore, attenuate the morphine withdrawal hyperexcitability. This is the very mechanism for the L-type calcium channel blockers such as nimodipine, nifedipine and nicardipine that were found to protect against naloxone-precipitated morphine withdrawal in mice and rats [5, 11, 12]. In addition to this, calcium channel blockers were shown to possess antinociceptive effects [13, 14] and also strengthen the acute central effects of opiates in animals [15, 16].

A second likely possibility is that opiate withdrawal results in augmented central release of glutamate, which in turn contributes to the signs typifying opiate withdrawal [17-19]. There are several lines of evidence indicating an important role for NMDA receptors in opiate tolerance, dependence and withdrawal [20]. All non-competitive NMDA receptor channel antagonists have been shown to attenuate the expression of withdrawal syndrome in morphine dependent mice [20]. Being a non-competitive antagonist for the NMDA receptor channel [9], magnesium could antagonize the action of glutamate and hence prevent the development of morphine tolerance and dependence. Magnesium alone has not been tested before for its action on morphine tolerance and dependence, but in one report, oral administration of magnesium in cocaine addicts was shown to decrease some cocaine withdrawal signs such as respiratory depression, dizziness and euphoria [21].

In agreement with previous findings [3], administration of calcium alone up to 50 mg/kg, did not affect the development of morphine tolerance and dependence. Magnesium alone on the other hand, worsened some and improved other signs of morphine withdrawal. It is, therefore, the mixture of calcium and magnesium at specific concentrations that produces appropriate effects. The calcium and magnesium contents of each CalMag capsules are 1,000 mg and 500 mg, respectively. The maximum tolerable doses of the mixture of calcium and magnesium in mice were 50 mg/kg and 25 mg/kg, respectively. Increases above these values caused serious overt reactions (hyperventilation, tremor, hyperactivity) and lower doses did not work as well. Since these capsules are designed for oral administration, the addition of calcium to the drug mixture, among other things, could improve the absorption of magnesium [22]. Further studies are underway to investi-
gate the chronic effects of CalMag on morphine tolerance and dependence.

**Conclusion**

Results show that CalMag administration could significantly prevent the development of morphine tolerance and dependence. The magnesium content of the CalMag capsules seem to play the pivotal role in attenuating the withdrawal signs as magnesium alone proved to be effective to a certain degree. Calcium alone, however, does not affect the state of morphine tolerance and withdrawal.

**References**


