Effects of chronic administration of calcium-magnesium soft gels on morphine tolerance and dependence in mice

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Abstract. The aim of present study was to assess the effects of chronic administration of calcium-magnesium soft gels on the development of morphine tolerance and dependence in mice. Tolerance was assessed using the tail-pinching test and withdrawal signs of morphine were precipitated by injecting naloxone 2 h after the final morphine injection. CalMag capsules were given in final doses of 50/25, 25/12.5 and 12.5/6.25 mg/kg based on calcium/magnesium ratio. Similar doses of Ca and/or Mg were prepared, separately. CalMag at 50/25, 25/12.5 mg/kg and the mixture of calcium and magnesium (Ca + Mg) at 50/25, 25/12.5, 12.5/6.25 mg/kg and calcium at 50, 25 mg/kg significantly reduced the number of jumps. The number of standings was only reduced after the administration of CalMag at 50/25 mg/kg and Ca + Mg at 25/12.5 mg/kg. The development of morphine tolerance was prevented in all drug-treated groups, except the one which received 6.25 mg/kg Mg. The data suggested that combination of calcium and magnesium at 50/25 and 25/12.5 mg/kg prevented the development of tolerance and dependence. It seems that other ingredients of CalMag capsules do not have an important effect on preventing tolerance and withdrawal signs. Compared to the acute effects, chronic administration of CalMag allowed the effective dose to be reduced. Unlike the acute treatment, chronic administration of calcium alone was effective in reducing morphine tolerance and dependence, and magnesium had no significant effect on withdrawal signs, suggestive of some pharmacological adaptations.

Key words: calcium-magnesium softgels, morphine, withdrawal, tolerance

Opioid analgesics such as morphine are widely used in treatment of acute and chronic pain but their clinical use is greatly limited by development of tolerance and dependence produced by prolonged administration [1, 2]. The overall intensity of physical dependence is gauged by the severity of the withdrawal syndrome.

A detailed understanding of the molecular and cellular mechanism of morphine tolerance and dependence is considered to be essential for treating and the prevention of these phenomena [1, 3, 4]. Based on the interaction between opiate and non-opiate receptor systems including N-methyl-D-aspartate (NMDA) [5-7], calcium channels [1, 8, 9],
gamma-aminobutyric acid (GABA) [10], and α-adrenergic receptor in the CNS [11], several drugs have been tested for their effects on the development of tolerance and physical dependence [11]. None of these drugs, however, have proven to be completely effective and without drawbacks. Calcium and magnesium have been shown to play an important role in morphine tolerance and dependence. Chronic morphine treatment has been shown to up-regulate calcium channels. The increase in intracellular Ca\(^{2+}\) and the consequent neuronal hyperactivity is related to the development of morphine tolerance and withdrawal syndromes [1]. NMDA receptor activation by chronic morphine treatment results in increased permeability of these receptors to Ca\(^{2+}\) ions and therefore, an increase in intracellular Ca\(^{2+}\) [5-7]. Magnesium as a NMDA receptor antagonist blocks the Ca\(^{2+}\) influx and attenuates the development of morphine tolerance and dependence [6, 12, 13]. Many studies have investigated the effect of calcium or magnesium separately, but the effect of these two elements in combination had not been studied. By administrating a combination of these two elements, besides getting the benefits of both for the body, a better effect on attenuating morphine tolerance and dependence may also be achieved.

Calcium magnesium soft gels (CalMag) are a good source 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. Calcium and magnesium supplements such as CalMag capsules are generally given to aid in the development and maintenance of bones [14]. Our previous study showed that the acute treatment of morphine tolerant and dependent mice with CalMag can significantly attenuate morphine tolerance and withdrawal syndromes [15]. The goal of the present study was to evaluate the effects of chronic administration of CalMag on the development of morphine tolerance and dependence in mice. With chronic administration it may be possible to reduce the effective dose of CalMag, and therefore, it could be used in the daily diet of morphine dependent and tolerant individuals. We also assumed that chronic use of CalMag would have a better effect on the attenuation of morphine tolerance and dependence. To eliminate the influence of other components of CalMag capsules, we compared the effect of CalMag with that of its calcium and/or magnesium content, separately.

### Materials and methods

#### Animals

Male NMRI mice (Pasteur institute, Tehran, Iran) weighing 25-30 g were housed in cages of six with controlled room temperature (22-25°C) in 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 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. 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 method of administration

Morphine sulfate (TEMAD, Iran), was dissolved in saline. Naloxone hydrochloride (TEMAD, Iran) was used to induce morphine withdrawal syndrome. Among other things, one capsule of CalMag (Bluebonnet Nutrition Corporation, Sugar Land, Texas, USA) containing 1 000 mg of calcium and 500 mg of magnesium was carefully suspended in 100 mL saline plus 0.5% Tween 80. This drug solution was brought up to appropriate concentration while warming to 40°C with vigorous shaking. Equal concentrations of calcium chloride (CaCl\(_2\).2H\(_2\)O) and magnesium chloride (MgCl\(_2\).6H\(_2\)O) were prepared in saline and used separately or mixed.

Three different doses (calcium/magnesium: 50/25, 25/12.5 and 12.5/6.25 mg/kg) of CalMag were used in our study. 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.

#### Tolerance paradigms

Groups of 6-13 mice were chosen randomly for each dose of drugs. The treatment schedule consisted of twice daily s.c. injections of 20 mg/kg morphine at 08:00 and 18:00 for 4 days and 20 mg/kg only at 08:00 on the 5\(^{th}\) day. CalMag, calcium and/or magnesium and vehicle were administered i.p. 30 min before morning injections of morphine for 4 days. The control mice (drug naïve) were treated with just saline (s.c. and i.p.).

Morphine antinociception lasts about 2 hours. Tolerance was assessed 30 min after the final mor-
phine injection based on loss of the antinociceptive effect of morphine during 5 days, using the modified Haffner's method, the tail-pinch test [16].

For the tail-pinch assay, a flattened clip (about 10 mm wide) was placed approximately 1 inch from the base of a mouse's tail, and only the mice that responded to the clip placement by turning back and biting the clip within 15 s were used in this test. Drug-naive mice responded to this pressure by immediately vocalizing and biting the clip. The mice treated by a single injection of morphine did not respond to the pain during 15 sec. The clip was never applied to the mouse's tail longer than this. An animal that did not respond before 15 s (cut-off time) was assigned a latency of 15 s. Response time to pain was reduced after development of morphine tolerance on day 5.

**Morphine withdrawal syndrome**

Groups of 6-9 mice were chosen randomly for each dose of drugs. Morphine was injected s.c. twice daily at 08:00 and 18:00 for 5 days as described by Itoh et al. with the following modifications [17]. Escalating doses, 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) were administered. CalMag, calcium and/or magnesium and vehicle were injected (i.p.) 30 min prior to morphine injections in the morning for 4 days.

Withdrawal signs were precipitated by injecting naloxone (5 mg/kg, i.p.) 2 h after the final injection of morphine. Immediately after a naloxone challenge, mice were individually placed in an observation box and withdrawal signs were evaluated during 20 min by counting the number of jumps and standings. Qualitative signs (hair raising, fast breathing, eye ptosis and diarrhea) were rated according to the Gellert and Holtzman rating scale [18]. This scale consists of graded signs and checked signs. Graded signs 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 t-test and one-way analysis of variance (ANOVA) with post-hoc Newman-Keuls test and expressed as mean ± S.E.M. Qualitative scores were analyzed with Mann-Whitney and expressed as median ± interquartile ranges. In all comparisons, p < 0.05 was considered significant.

**Results**

**Morphine tolerance**

The maximum tolerable dose of CalMag in mice was found to be 50/25 mg/kg and doses above this, caused severe overt reaction (e.g. tremor and hyperventilation). The tail-pinch test was used to investigate the effects of chronic administration of CalMag and calcium and/or magnesium on the development of morphine tolerance. In naïve mice (control group, n = 10) the response time to pain was 1.9 seconds (figure 1). As depicted in figure 1, administration of a single dose of morphine at 20 mg/kg produced an antinociceptive effect that was observed by the lack of response to exerted pain during the cut-off time (15 s). The mean response time to pain was gradually shortened from 15 s on the first day to 5.8 s on the 5th day in animals receiving salin (i.p.) and morphine (s.c.) which shows the development of tolerance (figure 1). With the exception of magnesium at 6.25 mg/kg, pretreatment with three doses of the test compounds significantly inhibited the development of morphine tolerance (p < 0.05) (figure 1). Chronic administration of magnesium at 50 mg/kg, was not tolerable in mice, and caused 4 out 6 mice to die. The effects of different doses of CalMag (50/25, 25/12.5 and 12.5/6.25) were also compared and, the results showed no significant differences between them.

**Morphine withdrawal signs**

In mice treated with morphine for 5 days, naloxone injections precipitated the standard behavioral signs of withdrawal. In the drug-naive group, however, the injection of naloxone did not trigger behavioral changes.

As illustrated in figure 2, pretreatment with CalMag (50/25 and 25/12.5 mg/kg), the same doses of calcium/magnesium mixture (Ca + Mg), and calcium (50 and 25 mg/kg) significantly reduced the number of jumps (p < 0.05). In addition, only the Ca + Mg at 12.5/6.25 had significant effects on number of jumps (p < 0.05) and unlike other treatments, magnesium alone at 6.25 mg/kg worsened the state of withdrawal by increasing the number of jumps, although this effect was not significant.

The number of standings was significantly reduced only by 50/25 mg/kg CalMag and 25/12.5 mg/kg Ca + Mg (p < 0.05) (figure 3). From comparison of different doses of CalMag (50/25, 25/12.5 and 12.5/6.25), only CalMag at 12.5/6.25 mg/kg was
ineffective in reducing the number of jumps and standings.

The effects of CalMag, calcium and/or magnesium at 50 and 25 mg/kg on 4 qualitative signs of morphine withdrawal are shown in table 1. The results showed that CalMag 50/25 and 25/12.5 significantly reduced most of these signs. None of the above test compounds at 12.5 mg/kg produced significant effects on the qualitative withdrawal signs and was therefore excluded from the table.

Figure 1. Effects of chronic administration of CalMag soft gels and calcium and/or magnesium on the development of morphine tolerance in mice. Drug naive mice responded to the pain immediately. Mice treated by a single injection of morphine (first day) did not respond to the pain during 15 sec (cut-off time). Morphine was given twice daily over a period of 5 days. Test compounds were injected prior to morphine injections for 4 days. Results are the mean (± SEM) from groups of 6-13 mice. * p < 0.05 for comparison between morphine plus test compounds with morphine alone on day five.

Figure 2. Effects of chronic administration of CalMag soft gels and calcium and/or magnesium on the number of jumps. Morphine was given in increasing doses over a period of 5 days. Test compounds were injected prior to morphine injections for 4 days. Naloxone was injected 2 hours after the final morphine injection, and the number of jumps was recorded for 20 minutes. Results are the mean (± SEM) from groups of 6-9 mice. * p < 0.05 for comparison between morphine plus test compounds with morphine alone.
Table 1. Effect of chronic administration of two different doses of CalMag and calcium and/or magnesium on naloxone-precipitated morphine withdrawal.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Fast breathing</th>
<th>Hair standing</th>
<th>Diarrhea</th>
<th>Ptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>4 (3.5-4)</td>
<td>3 (3-4)</td>
<td>3.5 (2.5-4)</td>
<td>3 (3-4)</td>
</tr>
<tr>
<td>CalMag (50/25)</td>
<td>2* (2-2.7)</td>
<td>2* (2-3)</td>
<td>2* (1.25-2.7)</td>
<td>2* (1.25-2.7)</td>
</tr>
<tr>
<td>Ca (50 mg/kg) + Mg (25 mg/kg)</td>
<td>2* (2-2)</td>
<td>2.5* (2-3)</td>
<td>2 (2-3)</td>
<td>3 (3-3)</td>
</tr>
<tr>
<td>Ca (50 mg/kg)</td>
<td>2* (1-3)</td>
<td>3 (3-3)</td>
<td>3 (3-3)</td>
<td>2* (2-2)</td>
</tr>
<tr>
<td>Mg (25 mg/kg)</td>
<td>3* (2-3)</td>
<td>2* (2-3)</td>
<td>2* (2-3)</td>
<td>2.5* (2-3)</td>
</tr>
<tr>
<td>Saline</td>
<td>4 (3-3)</td>
<td>3 (3-3)</td>
<td>4 (3.2-4)</td>
<td>4 (3-4)</td>
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<td>2* (2-2)</td>
<td>3 (2-3)</td>
<td>2.5* (2-3)</td>
<td>2.5* (2-3)</td>
</tr>
<tr>
<td>Ca (25 mg/kg) + Mg (12.5 mg/kg)</td>
<td>2.5 (1-3)</td>
<td>3 (2-3)</td>
<td>2* (2-3)</td>
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<td>3 (2-3)</td>
<td>3 (2-3)</td>
<td>2* (2-3)</td>
<td>2.5* (2-3)</td>
</tr>
<tr>
<td>Mg (12.5 mg/kg)</td>
<td>2.5 (2-3)</td>
<td>2.5 (2-3)</td>
<td>2.5* (2-3)</td>
<td>2* (2-3)</td>
</tr>
</tbody>
</table>

Values are expressed as median scores (± interquartile ranges in parenthesis, n = 6-9). Two different set of experiments, each with its own control (saline) group carried out on separate days. Morphine was given over a period of 5 days, test compounds were injected prior to morphine injections for 4 days. Naloxone was injected 2 hours after the final morphine injection, and the number of standings was recorded for 20 minutes. Results are the mean (± SEM) from groups of 6-9 mice. * p < 0.05 for comparison between morphine plus test compounds with morphine alone.

Figure 3. Effects of chronic administration of CalMag soft gels and calcium and/or magnesium on the number of standings. Morphine was given in increasing doses over a period of 5 days. Test compounds were injected prior to morphine injections for 4 days. Naloxone was injected 2 hours after the final morphine injection, and the number of standings was recorded for 20 minutes. Results are the mean (± SEM) from groups of 6-9 mice. * p < 0.05 for comparison between morphine plus test compounds with morphine alone.
Discussion

Previous results showed that acute administration of CalMag could significantly attenuate the development of morphine tolerance and dependence [15]. In line with these findings, the current study was designed to investigate the chronic effects of CalMag capsules. By chronic administration, it is possible to reduce the effective dose of the drug and therefore reduce any possible toxicity. To eliminate the influence of other components of CalMag capsules, we compared the effects of calcium and/or magnesium separately with that of CalMag on the development of morphine tolerance and dependence. The results of the tail-pinch assay showed that pretreatment with all the test compounds in different doses, except 6.25 mg/kg of magnesium, significantly reduced the development of morphine tolerance. With the exception of magnesium, the other test compounds significantly attenuated the signs (jumps and standing) of naloxone precipitated morphine withdrawal. In addition, most of qualitative signs of morphine withdrawal were also reduced by chronic administration of CalMag.

The mechanisms underlying the inhibitory effects of CalMag capsules on the development of morphine tolerance and dependence are not well known. The results of this study clearly showed that the effect of CalMag was mainly due to the calcium and magnesium contents of the capsules, as chronic administration of calcium and magnesium also prevented morphine tolerance and dependence. Chronic morphine treatment has been shown to up-regulate calcium channels. The increase in intracellular Ca\textsuperscript{2+} and the consequent neuronal hyperactivity is associated with the development of morphine tolerance and withdrawal syndrome [1]. Therefore in theory, calcium should potentiate the development of morphine tolerance and dependence, a fact that is in conflict with our findings. One possible explanation may be the probable down-regulation of calcium channels due to chronic calcium administration that reverses the effects of calcium channel up-regulation by chronic morphine treatment. Some experiments have investigated the effects of co-administration of calcium with morphine. Smith et al. found that calcium administration into the icv space of mice blocked opioid-induced antinociception [19]. In a previous study it was shown that acute administration of calcium did not attenuate morphine tolerance and dependence [15]. Various studies have investigated the effects of calcium channel antagonists on the development of morphine tolerance and dependence. The results are controversial. Seyler et al. found that verapamil increased morphine analgesic effects in mice [20]. Zarauza et al. observed no significant differences in morphine antinociception by administration of oral nifedipine or i.v. nimodipine, and, surprisingly, the nifedipine group had a larger morphine consumption during 24-48 hours than the control group [12]. It is probable that calcium channel antagonists are more effective by intrathecal and epidural routes as was demonstrated in the study of Choe et al. [21]. The other important underlying mechanism is NMDA receptor activation by chronic morphine treatment which results in increased permeability of these receptors to Ca\textsuperscript{2+} ions and therefore, an increase in intracellular Ca\textsuperscript{2+} [5-7]. Magnesium as a NMDA receptor antagonist blocks the Ca\textsuperscript{2+} influx and attenuates the development of morphine tolerance and dependence [6, 12, 13]. Magnesium-deficiency has been shown to cause hyperalgesia in rats, a phenomenon that was considered to be due to spinal NMDA receptor activation [22]. The effect of magnesium alone on morphine tolerance and dependence is somehow contradictory. The results of a study by Zarauza et al. showed no changes in postoperative morphine consumption by co-administration of magnesium sulfate (i.v.) and morphine [12]. Begon et al. found that the co-administration of magnesium (i.p.) and morphine (i.v.) potentiated the antinociception of morphine [13]. Kroin et al. found that intrathecal co-infusion of magnesium and morphine attenuated the development of morphine tolerance, and potentiated antinociception [23]. Nechifor et al. found that co-administration of magnesium acetate (i.p.) and morphine (i.p.) significantly reduced morphine withdrawal syndrome in rats [24]. McCarthy et al. found that intrathecal magnesium co-infusion with morphine prevented the development of morphine tolerance and reduced postoperative morphine consumption, but had no effect on reducing withdrawal signs [6], which is in agreement with our results. It is possible that in the case of chronic administration of magnesium, the affinity of NMDA receptors combined with Mg\textsuperscript{2+} has fallen to a certain degree.

In a previous study, we showed that acute treatment with CalMag capsules alleviated morphine withdrawal signs at 50/25 mg/kg [15]. In the present study, chronic administration of CalMag and especially the mixture of calcium and magnesium, at 50/25 mg/kg, caused overt reactions (weakness, tremor, hyperventilation and hypothermia), making the dose intolerable to animals. In this study, the
effective dose for chronic treatment with CalMag in preventing morphine tolerance and dependence was 25 mg/kg. Therefore, compared to acute doses, the effective dose of CalMag can be reduced by chronic administration. Also, in contrast to the results of the present study, acute administration of calcium was ineffective in reducing morphine tolerance and dependence, and acute magnesium could reduce the withdrawal signs. This comparison leads us to explain the adaptation of calcium channels and NMDA receptors as a possible consequence of long term drug treatment which affects the outcome [15]. Our results further highlight the fact that it is the calcium and magnesium content of the capsules that provide the necessary action for preventing tolerance and dependence and not the other contents of the capsules. Since these capsules are used as nutritional supplements, and so far no undesirable effects have been reported in humans, further studies could be designed to evaluate their effects in human morphine addicts.

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References


