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Enhanced Analgesic Effects of Tramadol and Common Trace Element Coadministration in Mice

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Chronic pain is managed mostly by the daily administration of analgesics. Tramadol is one of the most commonly used drugs, marketed in combination with coanalgesics for enhanced effect. Trace elements are frequent ingredients in dietary supplements and may enhance tramadol's analgesic effect either through synergic mechanisms or through analgesic effects of their own. Swiss Weber male mice were divided into nine groups and were treated with a combination of the trace elements Mg, Mn, and Zn in three different doses and a fixed dose of tramadol. Two groups served as positive (tramadol alone) and negative (saline) controls. Nociceptive assessment by tail-flick (TF) and hot-plate (HP) tests was performed at baseline and at 15, 30, 45, and 60 min after intraperitoneal administration. Response latencies were recorded and compared with the aid of ANOVA testing. All three trace elements enhanced tramadol's analgesic effect, as assessed by TF and HP test latencies. Coadministration of these trace elements led to an increase of approximately 30% in the average pain inhibition compared with the tramadol-alone group. The most effective doses were 0.6 mg/kg b.w. for Zn, 75 mg/kg b.w. for Mg, and 7.2 mg/kg b.w. for Mn. Associating trace elements such as Zn, Mg, and Mn with the standard administration of tramadol increases the drug's analgesic effect, most likely a consequence of their synergic action. These findings impact current analgesic treatment because the addition of these trace elements may reduce the tramadol dose required to obtain analgesia.

Key words: nociception; tramadol; magnesium; manganese; zinc; mice

Approximately 20% of Europe's adult population suffers from chronic pain, which has become a condition in itself (van Hecke et al., 2013). Therapeutic options for the treatment of chronic pain include life-style changes and interventions but, mainly, the regular administration of analgesics. Tramadol is a commonly prescribed, atypical, centrally acting, synthetic analgesic. In addition to its effect on μ-opioid receptors, tramadol inhibits the reuptake of both serotonin and norepinephrine, an effect that contributes to its analgesic efficacy (Grond and Sablotzki, 2004). Tramadol is commonly indicated in both acute and chronic pain states, ranging from trauma or renal colic to malignant pain (Khandave et al., 2010), and is frequently marketed in combination with acetaminophen, resulting in longer duration of action and enhanced analgesic effect (Dhillon, 2010).

Trace elements are essential chemical elements that act as cofactors of enzymes or are involved in metabolic processes. They are frequent ingredients in dietary supplements, which are widely consumed in the general population. Several studies have been performed to assess the potential beneficial role of dietary supplements, but the results have been controversial (Soni et al., 2010).

Zinc is an essential trace element found in high concentrations in the brain (Maserejian et al., 2012). Extracellular Zn has been shown to modulate membrane signaling and to influence NMDA receptors, which are known to play a role in pain transmission (Nozaki et al., 2011). Zn also plays an essential role in the downregulation of the neutral endopeptidase that degrades endogenous opioids (Kontargiris et al., 2012). Furthermore, Zn supplementation

SIGNIFICANCE:

To the best of our knowledge, this is the first study assessing the influence of trace elements (found in most dietary supplements) on analgesic drugs and pain. Demonstrating their ability to enhance tramadol's effects on pain and nociception in mice may represent a first step toward a clinical trial that will ultimately result in the improvement of pain management.

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may relieve pain in patients with burning mouth syndrome (Cho et al., 2010), and dietary Zn intake has shown pain-relieving effects (Nozaki et al., 2011).

Magnesium is the main intracellular divalent cation that influences the response of nerve tissue to stimuli (Biernat et al., 2014). It is involved in several metabolic processes, protein synthesis, and neuromuscular excitability (Laires et al., 2004) and may have a permissive effect on catecholamine's actions (Iannello and Belfiore, 2001). Mg also enhances endothelium-dependent vasodilatation and reduces inflammation. Severe Mg deficiency has been shown to affect oxidative metabolism adversely (Del Gobbo et al., 2013). The systemic administration of perioperative Mg has been shown to reduce postoperative pain and opioid consumption (De Oliveira et al., 2013).

Manganese plays an important role in enzyme activation. Both Mn catalase and Mn superoxide dismutase require this trace element, so Mn is involved in detoxification of superoxide free radicals and reducing oxidative stress (Martinez-Finley et al., 2013). Exposure to high concentrations of Mn has been shown to induce neurological anomalies such as behavioral changes, movement disorders, and muscle spasms (Kondakis et al., 1989). Mechanisms involved include 1) Mn's preferential uptake by the brain (Martinez-Finley et al., 2013), where it increases the rate of oxidative phosphorylation; 2) Mn's influence on the dopaminergic system (Bird et al., 1984); and 3) an increase in reactive oxygen species in the presence of high levels of Mn (Zwingmann et al., 2003).

Although dietary supplements are used by more than half the population, the potential interactions between trace elements and analgesic drugs are unknown. A study performed by our team in 2012 indicated a potential analgesic role of trace elements in an animal model (Tamba et al., 2013). As a second step in our investigation regarding the effects of trace elements on pain, this study assesses the influence of Zn, Mg, and Mn on tramadol-induced analgesia in rodents. To the best of our knowledge, there are no studies that assess the influence of trace elements (found in most dietary supplements) on analgesic drugs and pain. We hypothesize that, because of their influence on different parts of the nervous system, Mg, Zn, and Mn will enhance tramadol's analgesic activity.

MATERIALS AND METHODS

Animals

Adult Swiss Weber male mice (30–39 g) were used in the experiments. Animals were housed six per cage at 21°C ± 2°C under a 12-hr light/dark cycle with ad libitum access to food and water. Each mouse was habituated to the testing room for about 15 min prior to the beginning of the experiment. Animals were acquired and cared for in accordance with the NIH *Guide for the care and use of laboratory animals* and the Society for Neuroscience *Policies on the use of animals and humans in research*. The design of the experiment was approved by the University of Medicine and Pharmacy "Gr. T. Popa" Ethics Committee.

Drugs

The drugs used in these experiments were tramadol (KRKA Slovenia), Mg chloride ($MgCl_2$; Sigma-Aldrich ChimieGmbtH, Steinheim, Germany), Mn chloride ($MnCl_2$); Sigma-Aldrich), and Zn chloride ($ZnCl_2$; Sigma-Aldrich). All drugs were freshly diluted in normal saline and administered via the i.p. route.

Nociception Tests

The tail-flick (TF) test (D'Amour and Smith, 1941) assesses spinal response to pain by measuring the TF reflex latency following exposure to a heat stimulus. The TF unit (37360; UgoBasile, Gemonio, Italy) focuses a heat source on the distal portion of the mouse's tail (4–5 cm from the tip). The TF latency represents the reaction time necessary for the mouse to remove its tail from the heat source. Animals displaying baseline latencies of more than 7.5 sec were excluded from the study.

The hot-plate (HP) test, adapted from Woolfe and MacDonald (1944), assesses both spinal and supraspinal pathways. The mice were placed in an open Plexiglas tube on the HP apparatus (DS 37; UgoBasile) set at a temperature of 55°C ± 0.1°C, and the response latency was defined as the time between placing the animal on the plate and the occurrence of licking, shaking of hind paws, or jumping off the surface (nociceptive behavior). Animals displaying baseline latencies of more than 15 sec were excluded from the study.

Study Design

For each trace element, different doses were tested, chosen according to previous dose-dependent studies performed in our laboratory (Tamba et al., 2013). All substances were administered via the i.p. route. Groups of mice were injected with the following formulations: 0.9% saline for the negative control group (0.3 ml), tramadol 50 mg/kg b.w. for the positive control group, tramadol 50 mg/kg b.w. + $ZnCl_2$ (2.4, 1.2, or 0.6 mg/kg b.w.) for the Zn group, tramadol 50 mg/kg b.w.+ $MgCl_2$ (150, 75, or 37.5 mg/kg b.w.) for the Mg group, and tramadol 50 mg/kg b.w.+ $MnCl_2$ (14.4, 7.2, or 3.6 mg/kg b.w.) for the Mn group. All mice were assessed by means of the HP and TF tests before substance administration (baseline) and at 15, 30, 45, and 60 min after administration. Response latencies were automatically recorded.

Statistical Analysis

Data are expressed as mean ± SE. Statistical analysis was performed in Prism 5 (GraphPad, La Jolla, CA). Repeated-measures ANOVA was performed when appropriate. Post hoc comparisons of differences between individual groups were determined by the Turkey coefficient. Significance was set at $P < 0.05$. Pain inhibition (Wu et al., 2003) was calculated with the formula percentage inhibition = ([posttreatment latency − baseline latency]/[cut off latency − baseline latency]) × 100.

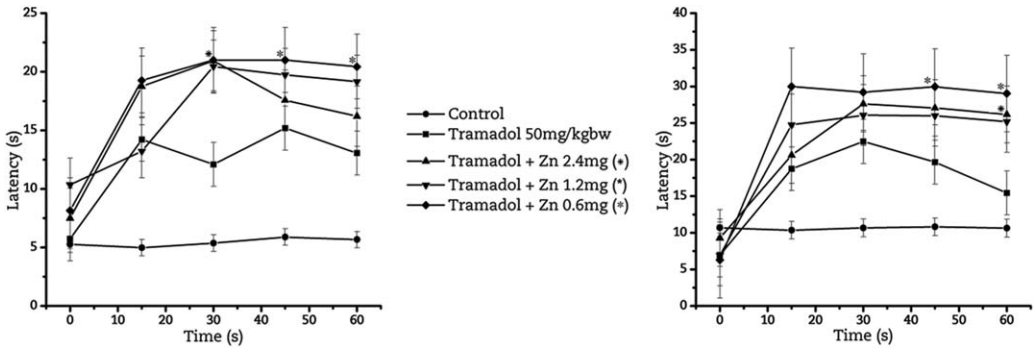

Fig. 1. Average latency values for Zn and tramadol coadministration. *$P < 0.05$ compared with tramadol alone.

RESULTS

Influence of Zn–Tramadol Administration on Acute Nociception

In the TF test (n = 6 mice/group), Zn significantly enhanced tramadol's analgesic effect 30 min after i.p. administration, with $P < 0.05$ (Turkey's post hoc analysis) for all three doses compared with tramadol alone. Repeated-measures ANOVA revealed a statistically significant substance effect of time ($F_{12,60} = 1.935$, $P = 0.0476$). In the large and medium-sized doses (2.4 and 1.2 mg/kg b.w., respectively), this effect was transitory, and although the latency response was longer in the Zn group, it no longer reached significance at 45 or 60 min. The tramadol group averages were 15.18 ± 1.93 sec at 45 min and 13 ± 2.17 sec at 60 min, whereas the Zn group averages were 17.57 ± 2.04 sec at 45 min and 16.20 ± 1.73 sec at 60 min for the 2.4-mg/kg b.w. dose and 19.73 ± 1.36 sec at 45 min and 19.15 ± 1.28 sec at 60 min for the 1.2-mg/kg b.w. dose. In contrast, the smallest dose (0.6 mg/kg b.w.) induced a long-lasting effect, with significant differences in latency at 30, 45, and 60 min compared with tramadol alone and average latencies of 21 sec at 30 and 45 min and 20.43 ± 0.42 sec at 60 min (Fig. 1). Compared with saline, all groups treated with the Zn–tramadol combination or tramadol alone had a statistically significant analgesic effect on the TF test that started after 15 min and persisted until the end of the experiment. All three doses induced an average pain inhibition of over 50% (76.95% for the 0.6-mg/kg b.w. dose, 58.54% for the 1.2-mg/kg b.w. dose, and 63.08% for the 2.4-mg/kg b.w. dose); tramadol's average pain inhibition in the TF test was 36.9% (Table I).

For the HP test (n = 6 mice/group), adding Zn had a similar effect on tramadol's analgesic effect with the exception that its onset was at 60 min for the 2.4- and 1.2-mg/kg b.w. doses, with an average of 15.52 ± 2.83 sec in the tramadol group vs. 26.17 ± 2.34 sec in the Zn 2.4-mg/kg b.w. group and 25.18 ± 2.58 sec in the Zn 1.2-mg/kg b.w. group ($P < 0.05$, Turkey's post hoc test). Repeated-measures ANOVA revealed a statistically significant substance effect of time ($F_{12,60} = 2.617$, $P = 0.0071$).

TABLE I. Average Pain Inhibition Values for Tested Substances in the TF Test After Administration of Trace Metals

Drug	0 min	15 min (%)	30 min (%)	45 min (%)	60 min (%)
Tramadol	0.00	50.79	39.01	48.04	46.66
Tramadol + $ZnCl_2$ 2.4 mg/kg b.w.	0.00	83.86	99.52	70.82	61.20
Tramadol + $ZnCl_2$ 1.2 mg/kg b.w.	0.00	27.47	99.52	86.18	83.53
Tramadol + $ZnCl_2$ 0.6 mg/kg b.w.	0.00	89.37	100.00	100.00	95.39
Tramadol + $MgCl_2$ 150 mg/kg b.w.	0.00	56.95	58.89	89.60	60.55
Tramadol + $MgCl_2$ 75 mg/kg b.w.	0.00	77.12	100.00	91.67	68.55
Tramadol + $MgCl_2$ 37.5 mg/kg b.w.	0.00	70.48	83.33	75.56	77.94
Tramadol + $MnCl_2$ 14.4 mg/kg b.w.	0.00	78.27	80.93	100.00	89.08
Tramadol + $MnCl_2$ 7.2 mg/kg b.w.	0.00	100.00	100.00	100.00	100.00
Tramadol + $MnCl_2$ 3.6 mg/kg b.w.	0.00	82.70	93.39	64.96	99.80
Saline	0.00	−2.01	0.71	7.30	3.77

In the 0.6-mg/kg b.w. group, the onset was earlier and persisted until the end of the experiment, with an average latency response of 29.95 ± 1.88 sec at 45 min and 29.02 ± 1.33 sec at 60 min, statistically different from the averages of the tramadol group (19.67 ± 3.02 sec at 45 min and 15.52 ± 2.83 sec at 60 min; Fig. 1). Compared with saline, all groups treated with the Zn–tramadol combination had a statistically significant analgesic effect on the HP test that started after 15 min and persisted until the end of the experiment. The tramadol group induced a significant analgesic effect on the HP test that lasted until 45 min. All three doses induced an average pain inhibition of over 60% (78.41% for the 0.6-mg/kg b.w. dose, 64.88% for the 1.2-mg/kg b.w. dose, and 62.40% for the 2.4-mg/kg b.w. dose); tramadol's average pain inhibition in the HP test was 41.74% (Table II). No statistically significant differences were noted among the groups at baseline assessment of TF and HP.

Influence of Mg–Tramadol Administration on Acute Nociception

The TF test (n = 6 mice/group) revealed that adding Mg did not produce a statistically significant enhancement of tramadol's analgesic effect for the 150-mg/kg b.w. dose. However, the average latency response was longer throughout the experiment in the Mg group. Both the intermediate and the small Mg doses induced significant changes after 30 min (with an average of 12.08 ± 1.87 sec in the tramadol group vs. 21.00 sec in both the 75-mg/kg b.w. and the 37.5-mg/kg b.w. Mg–tramadol groups; $P < 0.05$, Turkey's post hoc test). The 37.5-mg/kg b.w. Mg group had a more persistent increase in latency response, with statistically significant longer times that were also present 60 min after the i.p. administration (13.00 ± 2.17 sec in the tramadol group vs. 20.23 ± 1.89 sec in the Mg group; Fig. 2). Repeated-measures ANOVA revealed a statistically significant substance effect of time ($F_{16,80} = 2.766$, $P = 0.0014$). Compared with saline, all groups treated with the Mg–tramadol combination or tramadol alone had a statistically significant analgesic effect on the TF test that started after 15 min and persisted until the end of the experiment. All three doses induced an average pain inhibition of over 50% (61.46% for the 37.5-mg/kg b.w. dose, 67.47% for the 75-mg/kg b.w. dose, and 53.20% for the 150-mg/kg b.w. dose); tramadol's average pain inhibition in the TF test was below 40% (Table I).

TABLE II. Average Pain Inhibition Values for Tested Substances Assessed by the HP Test After Administration of Trace Metals

Drug	0 min	15 min (%)	30 min (%)	45 min (%)	60 min (%)
Tramadol	0.00	50.37	66.54	54.94	36.85
Tramadol + $ZnCl_2$ 2.4 mg/kg b.w.	0.00	54.03	89.30	86.18	82.48
Tramadol + $ZnCl_2$ 1.2 mg/kg b.w.	0.00	77.22	83.63	83.43	80.11
Tramadol + $ZnCl_2$ 0.6 mg/kg b.w.	0.00	100.00	96.74	99.81	95.51
Tramadol + $MgCl_2$ 150 mg/kg b.w.	0.00	29.70	41.52	49.04	33.90
Tramadol + $MgCl_2$ 75 mg/kg b.w.	0.00	83.75	90.69	94.90	99.29
Tramadol + $MgCl_2$ 37.5 mg/kg b.w.	0.00	89.57	89.96	88.64	75.81
Tramadol + $MnCl_2$ 14.4 mg/kg b.w.	0.00	72.68	86.65	86.57	78.90
Tramadol + $MnCl_2$ 7.2 mg/kg b.w.	0.00	82.30	84.56	96.22	90.38
Tramadol + $MnCl_2$ 3.6 mg/kg b.w.	0.00	89.57	89.96	88.64	75.81
Saline	0.00	−1.66	−0.31	0.55	−0.28

In the HP test (n = 6 mice/group), the 150-mg/kg b.w. Mg dose did not induce significant modifications throughout the experiment. The 75-mg/kg b.w. dose significantly enhanced tramadol's analgesic effect after 60 min, with an average latency response of 15.52 ± 2.83 sec in the tramadol group vs. 29.83 ± 2.63 sec in the Mg group ($P < 0.05$, Turkey's post hoc test). The average latency response time was longer in the 37.5-mg/kg b.w. Mg group compared with tramadol, although it did not reach statistical significance (Fig. 2). Repeated-measures ANOVA revealed a statistically significant substance effect of time ($F_{16,80} = 5.902$, $P < 0.0001$). Compared with saline, the 150-mg/kg b.w. Mg group did not induce significant changes. The tramadol group induced significant changes at 15, 30, and 45 min, but averages had a tendency to decrease in the tramadol group at 60 min. The small and intermediate Mg doses induced a statistically significant analgesic effect on the HP test that started after 15 min and persisted until the end of the experiment.

The intermediate and the small Mg doses induced an average pain inhibition of over 60% (68.80% for the 37.5-mg/kg b.w. dose and 73.73% for the 75-mg/kg b.w. dose). The 150-mg/kg b.w. dose induced a 33.90% average pain inhibition, smaller than the average for tramadol (41.74%); this difference, however, was not statistically significant (Table II). No statistically significant differences were noted among the groups at baseline assessment of TF and HP.

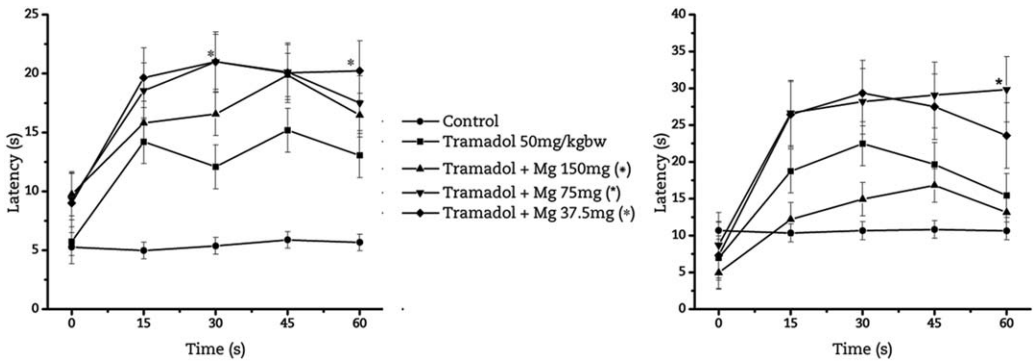

Fig. 2. Average latency values for Mg and tramadol coadministration. *$P < 0.05$ compared with tramadol alone.

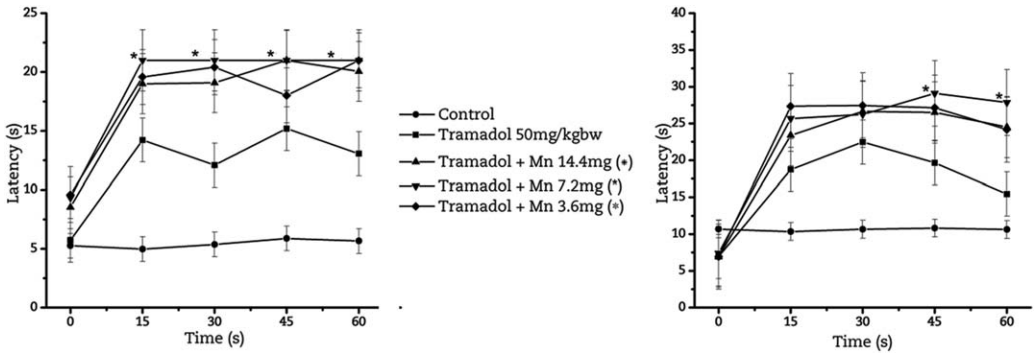

Fig. 3. Average latency values for Mn and tramadol coadministration. *$P < 0.05$ compared with tramadol alone.

Influence of Mn–Tramadol Administration on Acute Nociception

In the TF test (n = 6 mice/group), the intermediate dose induced the most persistent effect, with statistically significant differences (from the tramadol-alone group) in latency response time that started after 15 min and persisted until the end of the experiment (average values of 21 sec in the Mn–tramadol group at 15, 30, 45, and 60 min vs. 14.22 ± 1.94 sec, 12.08 ± 1.87 sec, 15.18 ± 1.93 sec, and 13.00 ± 2.17 sec in the tramadol group at the same times, respectively; $P < 0.05$, Turkey's post hoc test). Both the large and small doses of Mn increased tramadol's analgesic effect; in the high-dose group, the effect was statistically significant after 30 min, and in the low-dose group the effect was significant until 45 min (Fig. 3). Repeated-measures ANOVA revealed a statistically significant substance effect of time ($F_{16,80} = 4.967$, $P < 0.0001$). In comparison with the saline group, all groups treated with the Mn–tramadol combination or tramadol alone had a statistically significant analgesic effect on the TF test that started after 15 min and persisted until the end of the experiment ($P < 0.05$, Turkey's post hoc test). All three doses induced an average pain inhibition of over 60% (68.17% for the 3.6-mg/kg b.w. dose, 80.00% for the 7.2-mg/kg b.w. dose, and 69.65% for the 14.4-mg/kg b.w. dose); tramadol's average pain inhibition in the TF test was below 40%% (Table I).

In the HP test (n = 6 mice/group), neither the high nor the low Mn dose induced a statistically significant analgesic effect compared with tramadol alone. The intermediate dose (7.2 mg/kg b.w.) enhanced tramadol's effect 45 min after administration, with an average response latency of 29.10 ± 1.45 sec vs. 19.67 ± 3.02 in the tramadol-alone group. This effect persisted until the end of the experiment, with 27.85 ± 2.46 sec in the Mn–tramadol group vs. 15.52 ± 2.83 sec in the tramadol-alone group at 60 min (Fig. 3). Repeated-measures ANOVA revealed a statistically significant substance effect in time ($F_{16,80} = 6.630$, $P < 0.0001$). In comparison with saline, all Mn doses induced a statistically significant effect on the HP test that started after 15 min and persisted until the end of the experiment. All Mn doses induced an average pain inhibition of over 60% (68.80% for the 3.6-mg/kg b.w. dose, 70.69% for the 7.2-mg/kg b.w. dose, and 64.96% for the 14.4-mg/kg b.w. dose); the average pain inhibition of tramadol was 41.74% (Table II). No statistically significant differences were noted among the groups at baseline (T = 0) assessment of TF and HP.

DISCUSSION

The results of this study suggest that all three trace elements (Zn, Mg, and Mn) enhance tramadol's analgesic effect. Coadministration induced a statistically significant latency increase in both TF and HP tests compared with the tramadol-alone group. This result may be a consequence of modifications in the absorption and release rate of tramadol. A recent study tested the interaction between diclofenac and trace elements in an in vitro model of the gastrointestinal tract; the results showed that both Zn and Mg increased the drug's release rate, influencing the pharmaceutical availability in gastroresistant tablets (Biernat et al., 2014). In the current study, drugs were delivered via the i.p. route, which is somewhat similar to oral administration because of the absorption in the mesenteric vessels and hepatic metabolism (Turner et al., 2011). It is thus possible that the absorption process of tramadol is influenced by trace elements. A study with several animal models of pain and nociception (Tamba et al., 2013) indicated that some trace elements (such as Zn and Mg) may have an analgesic effect of their own, and another study showed that $MnCl_2$ administration in mice is associated with thermal analgesia (Tartau et al., 2006). With the two hypotheses taken together (enhancement of absorption and analgesic effect of trace elements on their own but with different mechanisms), a synergic effect between tramadol and trace elements is a likely explanation. Another possibility is an additive effect between tramadol and these trace elements, given that the main effect of tramadol is on μ-opioid receptors; some studies have explored the involvement of all three trace elements in the modulation of these receptors. However, published data in the field are rather scarce. To the best of our knowledge, this is the

first study assessing the influence of trace elements (found in most dietary supplements) on analgesic drugs and pain.

Zn

All doses of Zn enhanced tramadol's effect in both TF and HP tests, adding Zn to tramadol increased pain inhibition by almost 20% compared with the tramadol-alone group. The most effective dose seems to be the smallest one, 0.6 mg/kg b.w.; the onset of its effect was at 30 min in the TF test and at 45 min in the HP test. This suggests that Zn enhances tramadol's effect at both spinal and supraspinal levels; the 15-min difference between onset of effect in the HP and TF tests may be explained as the time necessary for Zn to cross the blood–brain barrier (BBB), indicating that the added effect is a consequence of Zn's own analgesic action and not its effect on tramadol metabolism.

Our data fall in line with previously published studies that have investigated the effect of Zn on pain. A study performed with mice indicated that NMDA receptors are highly sensitive to Zn and that Zn regulation plays an essential role in pain transmission and chronic pain development (Nozaki et al., 2011). Previous research on the analgesic effect of Zn alone has indicated that it acts both centrally and in the periphery (Tamba et al., 2013), suggesting that Zn enhances tramadol's analgesic effect by a synergic mechanism. In the spinal cord, Zn homeostasis has been shown to influence spinal cord recovery (Wang et al., 2011), and Zn dietary supplementation may regulate the expression of Zn transporter 1 in the dorsal horn of rats after spinal cord injury (Su et al., 2012). In the brain, Zn acts as a neuromodulator of glutamatergic transmission and antagonizes NMDA receptors (Ilouz et al., 2002). Some authors have suggested that it modulates γ-aminobutyric acid receptors, glycine, and adenosine triphosphate receptors (Smart et al., 2004; Hsiao et al., 2008) and influences pain through the nicotinic receptor ion channel (Hsiao et al., 2008) and that this influence can be either excitatory or inhibitory (Taly et al., 2009). Its deficiency is associated with inflammatory processes (Marcellini et al., 2006), and inflammation plays an important part in most pain processes.

Another possible explanation for Zn's influence on tramadol's analgesic abilities is its antidepressive effect. Current hypotheses for depression include anomalies in neurotransmission. Zn has shown antidepressant-like activity in animal models (Siwek et al., 2010) and has been shown to enhance traditional antidepressants' effect in animal models (Cunha et al., 2008) as well as in humans (Siwek et al., 2009). It is thus possible that a similar mechanism is involved in enhancing the effect of analgesics or that, through improvement of the depressive/mood, animals have a higher threshold for pain.

In the current study, the smallest dose of Zn induced the strongest antinociceptive effect. This can be explained by Zn's neurotoxic effects at high doses. As with most neuromodulators, its effect can be either excitatory or inhibitory, and this most likely depends on Zn's concentration (Nozaki et al., 2011). As the dose increases, the amplitude of Zn's effect decreases, and higher doses may even lead to hyperalgesia. The 0.6-mg/kg b.w. dose is higher than the recommended daily dose (6.4 mg/day or 5.7 mg/day in males or females, respectively; Lowe et al., 2013), but it is still in the same range (equivalent of 42 mg for a 70-kg person), whereas the intermediate and the high doses far exceed the daily recommended dose.

Mg

The small and medium-sized Mg doses enhanced tramadol's effect in both TF and HP tests, with an average increase of over 20% in pain inhibition compared with tramadol alone. Although the smallest dose (37.5 mg/kg b.w.) induced the most important increase in response latency in both tests, in the HP test this did not reach statistical significance. The intermediate dose (75 mg/kg b.w.) induced a significant change after 60 min in the HP test. Both doses induced significant changes after 30 min in the TF test. The large Mg dose induced no statistically significant enhancement of tramadol's analgesic effect as assessed by TF or HP. Even more, in the HP test, this dose decreased tramadol's average pain inhibition by approximately 8%. This suggests that Mg enhances tramadol's effect, especially in relatation to a spinal mechanism; the latency between onset of effects in the TF and HP tests can be a result of Mg's crossing of the BBB.

The relationship between Mg and pain has recently become a subject of great interest, with several studies indicating a potential role for Mg as a coanalgesic. A 2013 meta-analysis of randomized, controlled trials that included over 1,000 human participants revealed that perioperative Mg administration reduces both pain and opioid consumption (De Oliveira et al., 2013). Another study found that adding $MgSO_4$ to sufentanyl in the patient-controlled intravenous analgesia system enhanced analgesia in patients undergoing orthopedic surgery (Sedighinejad et al., 2014). Systemic administration of Mg has been shown to prolong anesthesia and duration of analgesia (Shukla et al., 2011), and concurrent administration of $MgSO_4$ decreased the rocuronium dose required for anesthesia (Kim et al., 2012). One study indicated that adding Mg to metamizol administration significantly decreased pain scores after tonsillectomy, indicating that Mg acts as a coanalgesic for metamizol (Tugrul et al., 2014).

A systematic review that underscored Mg's analgesic effect also indicated that it is most likely a consequence of an opioid-independent mechanism (Murphy et al., 2013), indicating that Mg's effect is most likely synergic with tramadol's. Other potential effects of Mg that may influence pain and pain transmission include 1) Ca channel inhibition (Dacey, 2001), 2) its effect on both pre- and postsynaptic neuromuscular transmission (Dubé and Granry, 2003) by decreasing the effects of acetylcholine on muscle receptors and increasing the threshold of axonal excitation, and 3) antagonism of the NMDA receptor.

In the current study, adding large-dose Mg to tramadol did not significantly influence tramadol's analgesic

effect; the tramadol-alone and tramadol + 150-mg Mg groups induced similar latency values. This is in accordance with our previous study (Tamba et al., 2013), in which we showed that the smaller 37.5-mg/kg b.w. dose, followed by the 75-mg/kg b.w. dose, had the best analgesic effects, whereas the 150-mg dose had a more moderate and less significant antinociceptive action. This could be explained by the facts that our dose was 30-fold higher than the average recommended daily dose (we administered 150 mg/kg b.w., and the daily recommended Mg requirement is 250–350 mg; Dubé and Granry, 2003) and that a possible CNS depressant activity at this dose could alter tramadol's effect.

Mn

Adding Mn to tramadol led to an increase in response latency in the TF test regardless of the Mn dose administered, although the intermediate dose (7.2 mg/kg b.w.) seems to be the most effective dose. Average pain inhibition was increased by more than 20% in all doses compared with tramadol. However, in the HP test, Mn proved less effective, and only the intermediate dose significantly enhanced tramadol's effect after 45 min; although the other doses also had average pain inhibition values larger than tramadol's, this did not reach statistical significance. These results suggest that, in addition to its central well-known site of action, Mn also acts at the spinal level. This hypothesis is supported by the fact that Mn supplementation enhanced tramadol's analgesic effect especially in the TF test, which assesses spinal pathways, and less so in the HP test, which evaluates both spinal and supraspinal pain transmission.

Few studies have explored the effects of acute Mn administration. However, chronic Mn exposure has been extensively studied because of its neurotoxicity, probably secondary to mitochondrial dysfunction and energy imbalance (Zwingmann et al., 2003). Consequences of chronic exposure are expressed especially in astrocytes and consist in neurodegeneration and neuroinflammation (Hazell, 2002), located especially in the supraspinal structures.

Some studies have indicated that Mn or other Mn-containing substances may have analgesic effects. A study performed on patients with degenerative joint disease who received a combination of several supplements that included Mn ascorbate indicated that knee osteoarthritis symptoms were relieved after this treatment (Leffler et al., 1999). However, the relationship between acute Mn administration and pain is unknown. One possible explanation for the analgesic effect of Mn could be its effect on Ca channels. An in vitro study (Tjalkens et al., 2006) indicated that subacute exposure to Mn inhibited Ca signaling in astrocytes because of its indirect effect on transient receptor potential (TRP) channel TRPC3. Both astrocytes and TRPC3 are also found in the spinal cord (Miyano et al., 2010) and are probably as sensitive to Mn as those in the brain. In the spinal cord, high levels of Mn have been shown to block synapses by interfering with nerve conduction along nerve fiber tracts (Bagust and Kerkut, 1980), and in the superior cervical ganglion Mn ions have induced a blockage of synaptic transmission. Furthermore, a paper by Gunter et al. (2006) hypothesized that Mn not only can inhibit Ca transporters but also can substitute for Ca in some transport processes. Because of the fact that Mn affects *supraspinal* Ca channels, we hypothesize that its analgesic effects could be a consequence of Mn's effect on *spinal* Ca channels; a reduction in Ca presynaptic influx may induce an apparent antinociceptive effect (Surprenant et al., 1990).

Study Limitations

This study has several limitations. Analgesia was assessed only by means of behavioral assessment. Only acute pain behavior was studied. Although no sedative or behavioral change effects were noted, motor behavior was not assessed by means of the rotarod test. The exact mechanisms by which the coadministration of trace elements enhances tramadol's effect have not been entirely explored and assessed. These findings have a potential impact on the results, and further research is required for translating these findings into clinical practice.

CONCLUSIONS

Our results indicate that associating trace elements such as Zn, Mg, and Mn with the standard administration of tramadol induces a more pronounced antinociception, as shown by increased response latencies in the TF and HP tests. Most likely, the effect is a consequence of synergic action between trace elements and tramadol. This may impact current analgesic treatment because adding inexpensive dietary supplements that contain these trace elements may help reduce the tramadol dose required to obtain analgesia.

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CONFLICT OF INTEREST STATEMENT

The authors have no identified conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: BT. Acquisition of data: TV, AM, TA. Analysis and interpretation of data: AM, TV, TA, BT. Drafting of the manuscript: TA, BT, AM, TV. Critical revision of the manuscript for important intellectual content: BT, TA. Statistical analysis: AM, TV. Obtained funding: BT, TA. Administrative, technical, and material support: BT. Study supervision: BT.

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