Antinociception Induced by Copper Salt Revisited: Interaction with Ketamine in Formalin-Induced Intraplantar and Orofacial Pain in Mice

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Aims: To evaluate in mice the antinociceptive effect of copper in spinal and trigeminal nociceptive pathways by using the intraplantar and orofacial formalin tests, respectively, and to examine whether this effect may interact synergistically with ketamine-induced antinociception. **Methods:** Nociceptive behaviors (licking/biting of the formalin-injected limb and rubbing/scratching of the formalin-injected orofacial area) in male mice were evaluated during a 45-minute observation period post–formalin injection. Dose-response curves for intraperitoneal (ip) copper sulfate and ketamine allowed their combination in equi-effective doses, and their interaction was determined with isobolographic analysis. The results were examined with one-way analysis of variance followed by the Bonferroni post hoc test. Significance was accepted at an alpha level of .05. **Results:** Irrespective of the region injected with formalin (upper lip or hindlimb), copper sulfate (0.3, 1.0, and 3.0 mg/kg) and ketamine (1.0, 3.0, and 10 mg/kg) dose-dependently decreased the nociceptive behaviors evoked by formalin injection. Isobolographic analysis showed a superadditive interaction between copper and ketamine at the spinal level, but this interaction was only additive at the trigeminal level. **Conclusion:** The results suggest that copper salts could be used to synergistically improve the efficacy of some commercial centrally acting analgesic agents, such as ketamine, while reducing the possibility of side effects. However, a synergistic effect probably should not be expected if treatment is for orofacial pain. *J Oral Facial Pain Headache 2018;32:247–257. doi: 10.11607/ofph.1961*

Keywords: *copper, intraplantar formalin, isobologram, ketamine, orofacial formalin*

Although trace elements such as copper, magnesium, and zinc are in very small amounts in the body, they are crucial for the health of living organisms and for the proper functioning of organs and metabolic processes.[1] As a divalent cation (Cu^{2+}), copper is the third most abundant dietary trace metal after iron and zinc and is present in a multiplicity of cells and tissues, with the highest concentrations in the brain and liver.[2] Copper accumulates in the brain and reaches extracellular concentrations of around 0.2 to 1.7 μM. The average copper concentration in the cerebrospinal fluid is approximately 70 μM, most of this being bound to proteins.[3] Interestingly, copper values are even higher in the synaptic cleft, reaching a Cu^{2+} concentration of about 100 μM.[4]

There are data indicating that copper may participate as a signaling molecule in the nervous system, as copper ions can decrease major components of neuronal activity, such as synaptic efficiency and neuronal excitability. In this regard, it has been shown that at a low micromolar concentration, Cu^{2+} can abolish synaptic long-term potentiation (LTP) in hippocampal slices[5,6] and cultured hippocampal neurons,[7] an effect that is in agreement with the blocking properties of copper on purinergic (P2X) receptors[8,9] and glutamate receptors.[10,11] On the other hand, it has been reported that at micromolar concentrations of 30 μm, Cu^{2+} can inhibit tetrodotoxin (TTX)-sensitive sodium channels in olfactory bulb neurons,[12] thereby decreasing their firing rate.[13] Furthermore, at these concentrations, copper blocks virtually all types of voltage-gated calcium channel isoforms expressed in neurons (T-, L-, N-, P-, and Q-type Ca^{2+} channels),[14] thus pointing to a general suppressive effect of copper

ions on neuronal excitability. However, caution should be exercised when interpreting these data, because at low micromolar concentrations copper has also demonstrated inhibitory effects on calcium-activated and inwardly rectifying voltage-gated neuronal potassium channels,[14] which are known to be involved in regulating the excitability of individual neurons.

There are some studies showing that copper has antinociceptive properties in various pain models. For instance, subcutaneous or oral administration of Cu^{2+}–based preparations had antinociceptive effects in the writhing test in mice and in the adjuvant arthritic rat pain model.[15] In a more recent study, intraperitoneal administration of incremental concentrations of Cu^{2+} chloride (0.5, 1.0, 2.0 mg/kg) and Cu^{2+} sulfate (0.5, 1.0 mg/kg) produced antinociceptive effects of about 30% in the hot plate and tail flick tests and of about 57% in the writhing test.[16] In addition, copper can also potentiate the effect of some conventional peripheral analgesic drugs; for example, the administration of Cu^{2+} increased the antinociceptive effect of fenoprofen, a nonsteroidal anti-inflammatory drug (NSAID), both in the acetic acid–induced writhing and the formalin acute pain models.[17] In another study, a significant analgesic effect of a Cu^{2+} complex was obtained in an arthritic rat model.[18] Thus, as a whole, these various data have revealed antinociceptive effects of copper in thermoalgesic and chemoalgesic acute tests.

However, the possibility of potentiating the activity of some classic centrally acting analgesics by the addition of copper has not yet been tested. The ultimate goal of drug combination is to obtain a higher therapeutic response with a reduction in the incidence and severity of side effects, which can be achieved by using lower doses of the drugs when a superadditive interaction between them is produced.[19,20] There is a current need for alternative nonopioid analgesics for the treatment of acute, chronic, and refractory pain. Ketamine, a fast-acting N-methyl-d-aspartate (NMDA) receptor antagonist, can provide safe and effective analgesia in different clinical settings. Ketamine has special indications for patients with opioid tolerance, acute hyperalgesia, and perioperative and neuropathic pain, as well as a role in the management of chronic pain, including both cancer and noncancer pain.[21] However, relatively large doses of ketamine produce dissociative effects, including induction of a psychedelic state causing agitation, hallucinations, and panic attacks,[22] thereby discouraging the use of ketamine alone as a therapeutic agent for chronic pain states that require long-term treatment. Knowledge of how the efficacy of ketamine could be potentiated without increasing its side effects could provide a good option for obtaining adequate analgesia with low, subdissociative doses of this drug. Thus, the aim of the present study was to evaluate in mice the antinociceptive effect of copper in spinal and trigeminal nociceptive pathways by using the intraplantar and orofacial formalin tests, respectively, and to examine whether this effect may interact synergistically with ketamine-induced antinociception.

Materials and Methods

Animals

A total of 133 naïve outbred CF1 male adult mice weighing 28 to 33 g were used for the study. Animals were housed six per cage and maintained in an environment with controlled temperature (21 ± 1°C) and light conditions (12/12 hours light/dark cycle, lights on at 7:00 am). Animals had ad libitum access to food and water and were allowed to habituate to the housing facility for 1 week before the beginning of the experiments. The experimental procedure was carried out during the light phase between 9:00 am and 12:00 am in a quiet room. The housing conditions and experimental procedures were approved by the Bioethics Committee of the Faculty of Medicine, University of Chile, and were in agreement with the ethical guidelines published by the International Association for the Study of Pain and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) in the USA.[23] To determine the number of required mice in each experimental group, a power analysis was conducted with the G*Power 3 Software.[24] All the experimental measurements were performed in a blinded condition. Each mouse was sacrificed at the end of the experiment by a carbon dioxide overdose.

Drugs

All animals received only one injection of a specific drug, drug combination, or solvent used. Copper sulfate pentahydrate ($CuSO_4$; Winkler) was dissolved in physiologic saline (0.9% NaCl) and administered via the intraperitoneal (ip) route in a volume of 0.5 mL at doses of 0.1, 0.3, 1, and 3 mg/kg for the intraplantar formalin test (n = 5 in each dose group) and at doses of 0.3, 0.6, 1, and 3 mg/kg for the orofacial formalin test (n = 5 mice in each dose group). Ketamine (Imalgene 1,000) was administered at doses of 0.3, 1, 3, and 10 mg/kg (ip) for both the intraplantar (n = 5 in each dose group) and the orofacial formalin test (n = 5 in each dose group). Two control groups (intraplantar formalin test = 7; orofacial formalin = 6) received a similar ip volume (0.5 mL) of the solvent used to dissolve $CuSO_4$ or ketamine (0.9% saline). Thus, 80 mice were given a single dose of a determined drug dose ($CuSO_4$ or ketamine), and 13 mice received the solvent and served as controls. Another

40 mice received a single injection of one out of four equi-effective combinations of $CuSO_4$ and ketamine, as indicated below in Isobolographic Analysis.

Behavioral Assessment

The animals were acclimatized in the experimental room 2 hours before the beginning of the experiments. At 15 minutes before the behavioral evaluation, mice were given a single injection of physiologic saline (control), $CuSO_4$ alone, ketamine alone, or $CuSO_4$ + ketamine. For behavioral testing, mice were placed into an acrylic cylinder (25 cm high × 25 cm in diameter) enclosed by two mirrors placed perpendicular to each other. Prior to testing, each mouse was placed into the cylinder for 10 minutes to acclimatize and minimize stress. Mice were then gently restrained and received 20 µL of 2% formalin solution injected into either the plantar surface of the right hindlimb or into the right whisker pad (lip) of the orofacial region.

Formalin Test

For the intraplantar formalin test,[25] the nociceptive behavior evaluated was licking/biting of the injected limb, and for the orofacial formalin test,[26] rubbing/scratching of the formalin-injected orofacial area (using either the ipsilateral fore- or hindpaw) was assessed. Both tests were run during a 45-minute observation period starting from the time of formalin administration and divided into 9 blocks of 5 minutes each. A nociceptive score was determined for each block by measuring the number of seconds that the animal exhibited nociceptive behavior.

The time course of the nociceptive response to formalin is usually studied by plotting the individual nociceptive scores obtained during the first 10 minutes following formalin injection (Phase 1) and between 10 and 45 minutes after formalin injection (Phase 2) for both the intraplantar[25,27,28] and orofacial[26,29,30] formalin tests. Nevertheless, preliminary statistical analyses of experimental data revealed that both $CuSO_4$ and ketamine similarly affected behaviors in Phase 1 and Phase 2 (ie, the effective dose that produced 50% of the maximal effect [ED_{50}] for each drug was not significantly different when comparing the antinociceptive effects between Phase 1 and Phase 2; for explanation, see below). Therefore, the data were analyzed for the total time of observation (1 to 45 minutes). By summing the nine individual nociceptive scores (NS) recorded during the total time of observation, a global nociceptive score (ΣNS) was obtained. This was subsequently used to calculate the antinociceptive effect of each dose of each drug as:

$$\text{Antinociceptive effect (\%)} = \left(\frac{[\Sigma\text{NSsaline} - \Sigma\text{NSdrug}]}{\Sigma\text{NSsaline}}\right) \times 100$$

. . . where ΣNSsaline is the algebraic sum of the scores under saline and ΣNSdrug is the algebraic sum of the scores under drug. Plotting the antinociceptive effect (%) against log dose allowed for obtaining the ED_{50} by linear regression analysis.

Isobolographic Analysis

Evaluation of the interaction of $CuSO_4$ and ketamine was performed with isobolographic analysis.[21,22,31] The isobologram is a graphic method that involves calculating the theoretical additive dose for each level of effect and statistically comparing it to the combination dose that causes the same effect experimentally. Equi-effective doses of both drugs alone are necessary to calculate the expected dose in a combination. To this end, each drug dose that produced ED_{50} was defined by using a linear regression analysis from the dose-response curve of four increasing doses of $CuSO_4$ and ketamine. Once the ED_{50} of each drug was obtained, a graph was constructed by placing the ED_{50} of $CuSO_4$ on the x axis and the ED_{50} of ketamine on the y axis. The union of the two points by a straight line (isobolo), also known as the line of additivity, allowed establishing the type of interaction of both drugs. Then, a dose-response curve for the coadministration of $CuSO_4$ + ketamine was carried out by administering the combination in fixed ratios of 1/2, 1/4, 1/8, and 1/16 of their respective ED_{50} values (ie, by combining the respective ED_{25}, $ED_{12.5}$, $ED_{6.25}$, and $ED_{3.125}$ values of $CuSO_4$ and ketamine). Each combination of $CuSO_4$ + ketamine was administered as a single (ip) injection 15 minutes before the intraplantar or the orofacial formalin injection. The relationship between the experimental value (experimental ED_{50}) of the combination and the theoretical value (theoretical additivity ED_{50}) determines the type of interaction: if the value is located under the line of additivity and is statistically different from the theoretical value, then the interaction is synergistic or superadditive (effect greater than the sum of the individual effects of each drug), and if it is not statistically different from the theoretical value, the interaction is simply additive (effect equal to the sum of the individual effects of each drug); conversely, if the experimental value is located above the line of additivity and statistically different from the theoretical value, it is a subadditive or antagonistic interaction. This relation can be calculated by the interaction index (γ = experimental ED_{50}/theoretical additive ED_{50}) between both drugs. When smaller than 1, the index corresponds to a synergistic interaction; when equal to 1, it corresponds to an additive interaction; and when greater than 1, it corresponds to an antagonistic interaction. Figure 1 shows a graphic illustration of the isobolographic method.

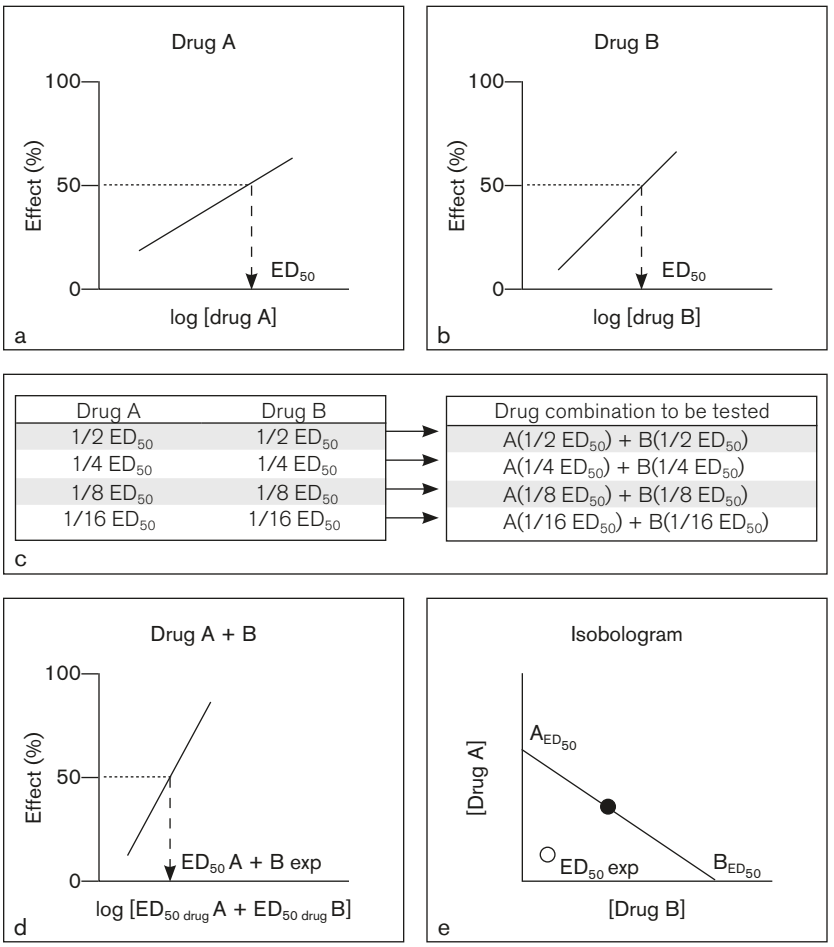

Fig 1 The isobolographic methods for analyzing the interaction of two drugs producing the same biologic effect. **(a, b)** Regression lines plotted from the dose-response data on the effects of drugs A and B on the same biologic parameter (pain threshold), allowing their respective ED_{50} values to be calculated. **(c)** Equi-effective doses of the drugs A and B to be combined (eg, 1/2, 1/4, 1/8, and 1/16 of their respective ED_{50} values) and later tested (on pain threshold) in a separate experiment. **(d)** Regression line obtained from the dose-response experiment using the equi-effective doses of the A + B drug combination, from which the experimental ED_{50} of the combination can be calculated. **(e)** An isobologram is plotted showing the line of additivity, which theoretically results from the combination of different proportions of the drugs, provided the interaction is additive. On the middle part of this line is the theoretical additive ED_{50} of the combination against which the experimental ED_{50} can be statistically compared. All data calculated are mean ± SEM values, and therefore error bars for both the experimental and the theoretical additive ED_{50} could be depicted. When the experimental ED_{50} / theoretical additive ED_{50} (eg, the γ index) is smaller than 1, it corresponds to a synergistic interaction; when equal to 1, it corresponds to an additive interaction; when greater than 1, it is an antagonistic interaction.

Data Analyses

The results of the scores obtained were expressed as means ± standard error of the mean (SEM), and the computed ED_{50} values included the 95% confidence intervals (CI). To characterize the interaction between the drugs studied, an isobolographic analysis was performed using a custom Microsoft Excel macro program based on the method previously described[21,22,31] and the interaction index was calculated. The results were examined using Student *t* test for unpaired data. To compare the effects of the different doses of each drug and their combinations, the results were examined using one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc multiple comparisons test. The statistical analyses were conducted with Prism 7.0 Software (GraphPad Software). Significance was accepted at an alpha level of .05.

Results

Antinociceptive Effect in the Intraplantar Formalin Test

Intraplantar administration of 2% formalin in control mice (20 µL) induced a score of nociceptive licking/biting behavior amounting to 220.9 ± 6.9 seconds for total time of observation (n = 7; Fig 2a). Administration of $CuSO_4$ induced a dose-dependent reduction of the nociceptive response induced by 2% formalin (Fig 2a). For total time of observation, licking/biting behavior scores amounted to 199.0 ± 4.0 seconds, 157.8 ± 4.4 seconds, 113.6 ± 4.7 seconds, and 93.1 ± 3.3 seconds for doses of 0.1, 0.3, 1, and 3 mg/kg of $CuSO_4$, respectively (Fig 2b). All these nociceptive scores were significantly lower than those obtained after physiologic saline administration ($P < .001$).

Ketamine administration also induced a dose-dependent reduction of the nociceptive scores induced by formalin (Fig 2c). For total time of observation in the formalin test, nociceptive scores were 197.4 ± 6.9 seconds, 166.0 ± 10.6 seconds, 126.2 ± 4.2 seconds, and 100.2 ± 6.2 seconds for doses of 0.3, 1, 3, and 10 mg/kg of ketamine, respectively (Fig 2d). The three higher doses of ketamine produced significantly lower nociceptive scores compared to physiologic saline ($P < .001$).

The administration of combinations of $CuSO_4$ + ketamine in equi-effective proportions of their respective ED_{50} values induced a dose-dependent reduction of the nociceptive behavior scores (Fig 2e). For total period of

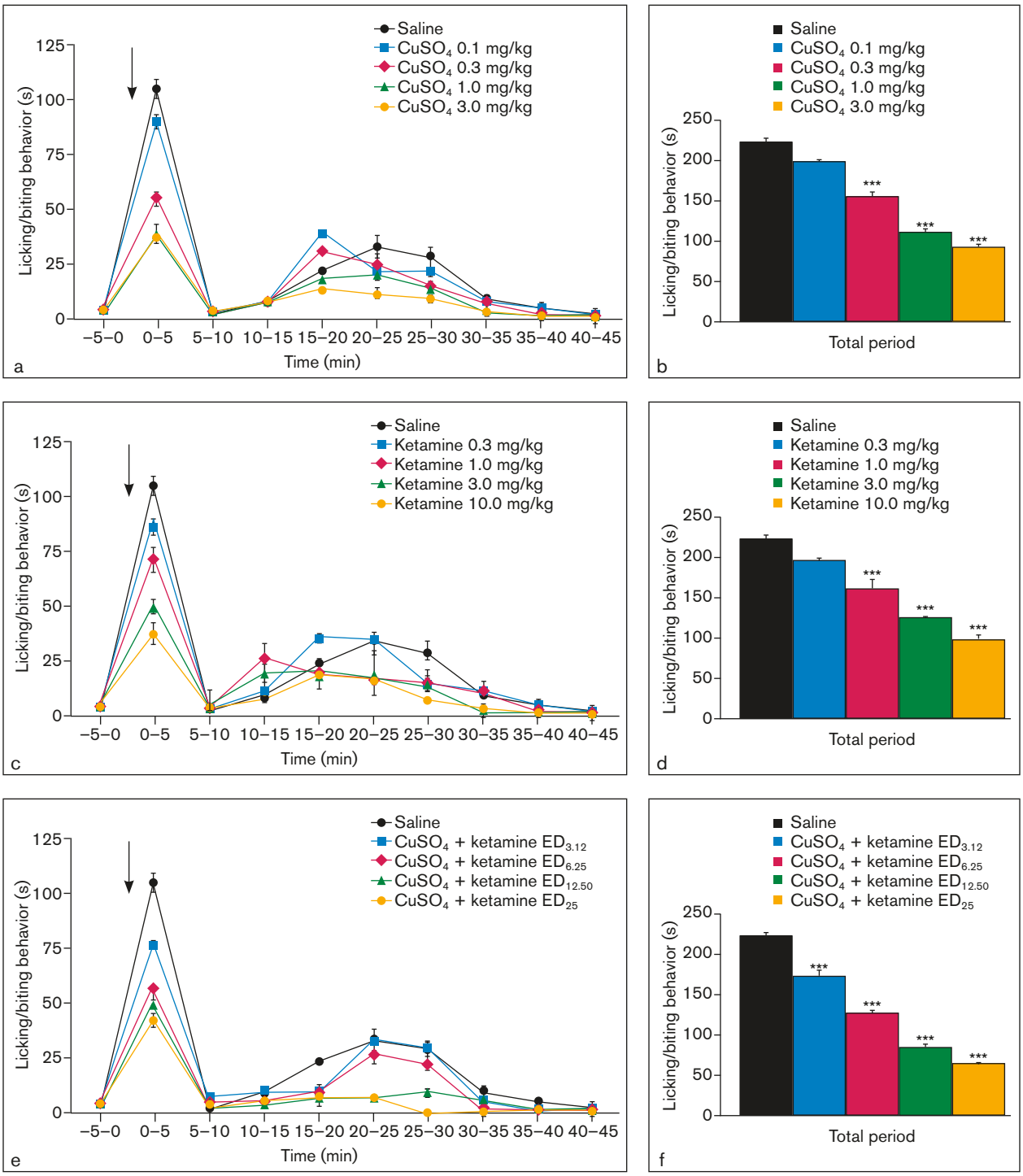

Fig 2 Effect of intraperitoneal (ip) administration of saline, $CuSO_4$, ketamine, or $CuSO_4$ + ketamine combination on nociceptive response, expressed as licking/biting behavior, elicited by intraplantar administration of 2% formalin. $CuSO_4$, ketamine, or $CuSO_4$ + ketamine combination were administered as a single ip injection 15 minutes before intraplantar formalin administration. **(a)** Time course of effects of physiologic saline and 0.1, 0.3, 1, or 3 mg/kg $CuSO_4$. **(b)** Global nociceptive score (ΣNS) for total time of observation after administration of physiologic saline or increasing doses of $CuSO_4$. **(c)** Time course of effects of physiologic saline and 0.3, 1, 3, or 10 mg/kg of ketamine administration. **(d)** ΣNS for total time of observation after administration of physiologic saline or increasing doses of ketamine. **(e)** Time course of effects of physiologic saline and $CuSO_4$ + ketamine combinations in proportions of their respective $ED_{3.125}$, $ED_{6.25}$, $ED_{12.5}$, and ED_{25} values. **(f)** ΣNS for total time of observation after ip administration of physiologic saline or increasing equi-effective doses of $CuSO_4$ + ketamine combination. Arrows indicate formalin injection. Each bar represents the mean ± SEM of five independent determinations. Intergroup statistics were compared using one-way ANOVA followed by Bonferroni multiple comparisons post hoc test (***$P < .001$).

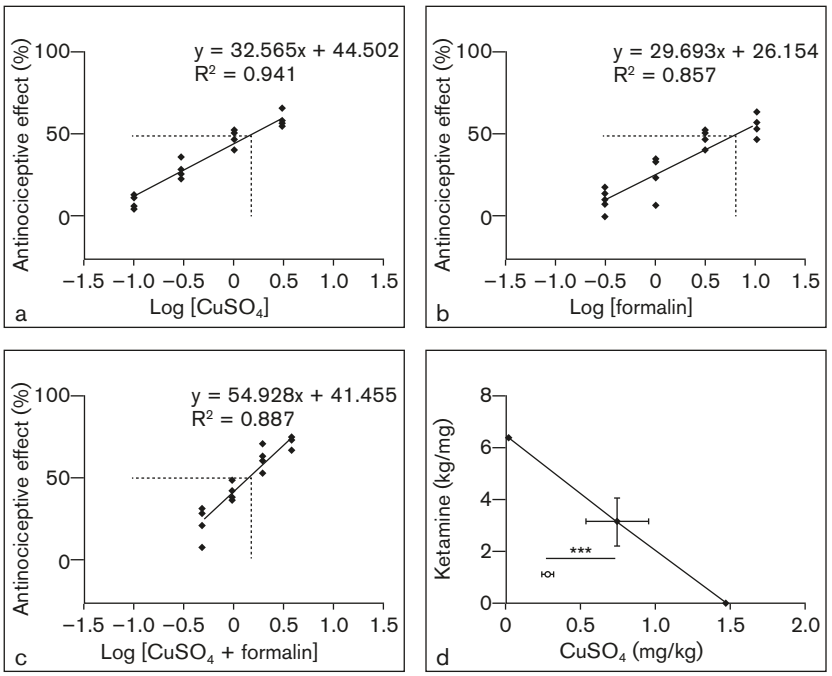

Fig 3 Dose-response data representing the antinociceptive effect (%) of **(a)** $CuSO_4$, **(b)** ketamine, and **(c)** $CuSO_4$ + ketamine combination in naïve mice submitted to intraplantar formalin testing, expressed as dose logarithm. The respective ED_{50} values were calculated from the regression lines and are shown in each figure with a segmented line. **(d)** Isobologram of interaction for $CuSO_4$ + ketamine combination in naïve mice for total observation period in the intraplantar formalin test. The black circle on the straight line represents the point of theoretical additivity of the combination, and the white circle corresponds to the experimental point. The experimental point was significantly different from the theoretical point (mean ± SEM; ***P < .001, two-tailed Student t test), indicating a superadditive interaction.

observation, association of $CuSO_4$ + ketamine administered in proportions of their respective $ED_{3.125}$, $ED_{6.25}$, $ED_{12.5}$, and ED_{25} values produced nociceptive scores of 172.8 ± 8.8 seconds, 126.2 ± 5.6 seconds, 84.6 ± 6.3 seconds, and 65.0 ± 3.8 seconds, respectively (Fig 2f). All the $CuSO_4$ + ketamine proportions administered led to significantly lower nociceptive scores compared to physiologic saline (P < .001).

The ED_{50} value for $CuSO_4$ was 1.48 mg/kg (95% CI: 1.21 to 1.79 mg/kg) (Fig 3a); the ketamine ED_{50} was 6.36 mg/kg (95% CI: 4.10 to 9.86 mg/kg) (Fig 3b); and the experimental ED_{50} for $CuSO_4$ + ketamine was 1.43 mg/kg (95% CI: 1.24 to 1.65 mg/kg) (Fig 3c). Isobolographic analysis for the administration of $CuSO_4$ + ketamine showed that the experimental ED_{50} was significantly lower than the theoretical additive ED_{50} (interaction index g = 0.365), which represents a superadditive effect (Fig 3d).

Antinociceptive Effect in the Orofacial Formalin Test

Orofacial administration of 2% formalin induced a rubbing/scratching behavior score of 183.5 ± 9.7 seconds for total time of observation (n = 6, Fig 4a). Administration of $CuSO_4$ induced a dose-dependent reduction of nociceptive rubbing/scratching behavior scores induced by 2% formalin (Fig 4a). Comparison of area under the curve (AUC) values showed that for the total period of observation, the three higher doses of $CuSO_4$ (0.6, 1, and 3 mg/kg) significantly reversed (P < .001) the development of hyperalgesia compared to physiologic saline ($CuSO_4$: 125.0 ± 11.0 seconds, 90.4 ± 6.8 seconds, and 69.8 ± 5.2 seconds, respectively; saline: 183.5 ± 9.7 seconds; Fig 4b). However, the lowest dose of $CuSO_4$ (0.3 mg/kg) did not significantly modify the rubbing/scratching behavior caused by formalin (155.0 ± 5.6 seconds).

As illustrated in Fig 4c, ketamine administration induced a dose-dependent reduction of nociceptive rubbing/scratching behaviors evoked by orofacial formalin. Comparison of AUC values showed that for total time of observation in the orofacial formalin test, the three higher doses of ketamine (1, 3, and 10 mg/kg) significantly reduced the nociceptive scores (ketamine: 148.8 ± 4.0 seconds, 101.6 ± 4.4 seconds, and 80.4 ± 4.2 seconds, respectively; P < .001) (Fig 4d).

Administration of $CuSO_4$ + ketamine in equi-effective proportions of their ED_{50} values induced a dose-dependent reduction of the nociceptive behavior produced by orofacial formalin (Fig 4e). For total period of observation, the association between the mix of $CuSO_4$ + ketamine in proportions of their respective $ED_{12.5}$, ED_{25}, and ED_{50} values decreased the nociceptive scores significantly (123.4 ± 6.4 seconds, 90.3 ± 8.1 seconds, and 77.9 ± 5.1 seconds, respectively; P < .001; Fig 4f).

The ED50 for $CuSO_4$ was 1.40 mg/kg (95% CI: 1.02 to 1.91 mg/kg) (Fig 5a); for ketamine, the ED_{50} was 5.73 mg/kg (95% CI: 4.26 to 7.72 mg/kg) (Fig 5b); for $CuSO_4$ + ketamine, the experimental ED_{50} was 4.47 mg/kg (95% CI: 3.62 to 5.51 mg/kg) (Fig 5c). The isobologram obtained for the orofacial formalin test indicated that the experimental ED_{50} was not significantly different from the theoretical additive ED_{50} (interaction index of 1.254), indicating that for the combination of $CuSO_4$ + ketamine, the interaction was only additive (Fig 5d).

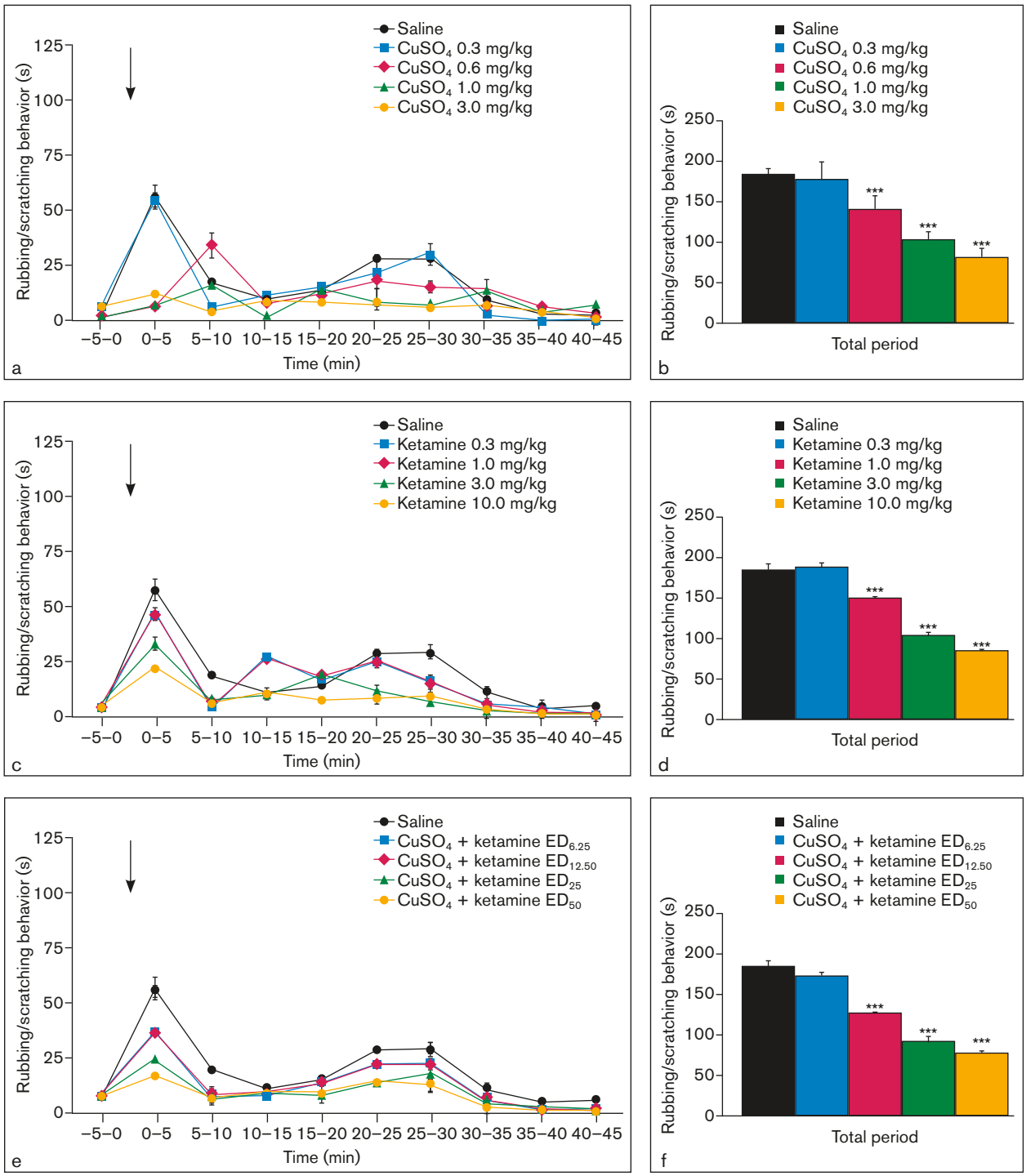

Fig 4 Effect of intraperitoneal (ip) administration of physiologic saline, $CuSO_4$, ketamine, or $CuSO_4$ + ketamine combination on nociceptive response, expressed as rubbing/scratching behavior, elicited by orofacial administration of 2% formalin. $CuSO_4$, ketamine, or $CuSO_4$ + ketamine combination were administered as a single ip injection 15 minutes before orofacial formalin administration. **(a)** Time course of effects of saline and 0.3, 0.6, 1, or 3 mg/kg $CuSO_4$. **(b)** ΣNS for total time of observation in orofacial formalin test after ip administration of physiologic saline or increasing doses of $CuSO_4$. **(c)** Time course of effects of physiologic saline and 0.3, 1, 3, or 10 mg/kg of ketamine administration. **(d)** Global nociceptive score (ΣNS) for total time of observation in orofacial formalin test after administration of physiologic saline or increasing doses of ketamine. **(e)** Time course of effects of saline and $CuSO_4$ + ketamine combinations in proportions of their respective $ED_{6.25}$, $ED_{12.5}$, ED_{25}, or ED_{50} values. **(f)** ΣNS after administration of physiologic saline or increasing equi-effective doses of $CuSO_4$ + ketamine combination. Arrows indicate formalin injection. Each bar represents the mean ± SEM of five independent determinations. Intergroup statistics were compared using one-way ANOVA followed by Bonferroni's multiple comparison post hoc test (*$P < .05$; ***$P < .001$).

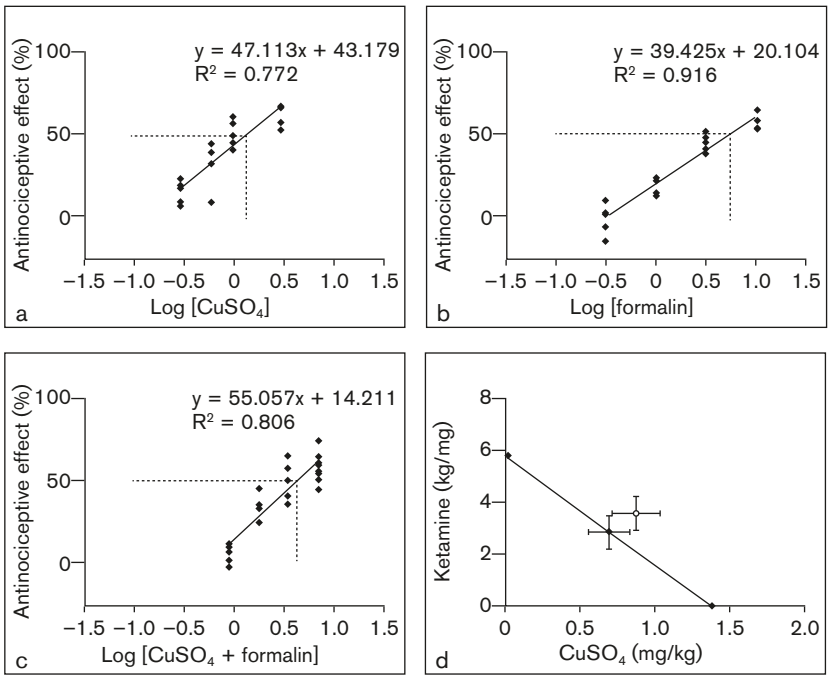

Fig 5 Dose-response data representing the antinociceptive effect of **(a)** $CuSO_4$, **(b)** ketamine, and **(c)** $CuSO_4$ + ketamine combination in naïve mice submitted to orofacial formalin testing, expressed as dose logarithm. The respective ED50 values were calculated from the regression lines and are shown in each figure with a segmented line. **(d)** Isobologram of interaction for $CuSO_4$ + ketamine combination in naïve mice for total observation period in the orofacial formalin test. The black circle on the straight line represents the point of theoretical additivity of the combination, whereas the white circle corresponds to the experimental point. The experimental point was not significantly different from the theoretical point (mean ± SEM; two-tailed Student *t* test), indicating an additive interaction of both drugs.

Discussion

The present results have shown that $CuSO_4$ produced a dose-dependent antinociceptive effect in the two mouse models of formalin-induced pain used in this study. This is in agreement with expectations, since a previous report showed that copper salts induced dose-dependent antinociception in mice in both the hot-plate and writhing algesimetric tests.[16] Furthermore, the antinociceptive effect produced by $CuSO_4$ also coincides with that obtained in other studies[17,32] in which copper NSAID complexes were found to produce higher antinociception than NSAIDs alone in the paw formalin test. In one of these studies,[17] the daily copper doses were higher than those used in the present study and were chronically given during a 28-day period. Some renal side effects were reported; however, these mostly corresponded to well-known side effects of the NSAID when used long-term.[17] The present study also showed that ketamine alone and the $CuSO_4$ + ketamine combination produced dose-dependent antinociception in both models of formalin-induced pain. It has been reported that subanesthetic doses of the NMDA antagonist ketamine induce antinociception in several pain models[33] and can decrease the hyperalgesia and allodynia present in chronic pain complaints.[19,34]

The main result of the present study was that $CuSO_4$ and ketamine interacted synergistically in the intraplantar formalin pain model, which means that there was a potentiation of the antinociceptive effect of the drugs. The ED_{50} of ketamine alone was 6.36 mg/kg, while the addition of $CuSO_4$ to ketamine (in proportions of each ED_{50}) lowered the ED_{50} for the combination to 1.43 mg/kg. The superadditive interaction between $CuSO_4$ and ketamine detected by isobolographic analysis upon intraplantar formalin testing originated from parallel regression lines obtained in the dose-response plots of the individual drugs, indicating that the potency ratio for these two drugs remained constant during testing of formalin-induced pain in normal rats.[21,22,31] Theoretically, superadditivity in the effects of two simultaneously administered antinociceptive drugs implies that the combined molecules act on anatomically and/or functionally different substrates for nociceptive processing, which may represent different neurons, different receptors in the same neuron, or even different sites of binding in the same receptor. In this regard, it is likely that Cu^{2+} had synergized the blocking effect of ketamine on the NMDA receptor channel by binding to an NMDA receptor site other than that bound by ketamine. This is in agreement with previous reports demonstrating that Cu^{2+} acts as a high-affinity NMDA receptor antagonist characterized by a voltage-independent mechanism of action,[10] whereas ketamine binds to the phencyclidine NMDA receptor site in a voltage-dependent fashion, causing a selective block of only open NMDA receptor channels.[35,36] A similar superadditive interaction for the antinociceptive effect of two NMDA receptor antagonists acting in different sites of the receptor has already been reported in rats, where the NMDA receptor antagonists ketamine and ± 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) act on the phencyclidine binding site and on the NMDA recognition competitive site, respectively.[37]

Nevertheless, synergy by Cu^{2+} of the antinociceptive effect of ketamine in the intraplantar formalin test could also be the result of a modulating effect of Cu^{2+} on other molecules that are different from NMDA receptors that are involved in nociceptive transmission/control and are known to be functionally affected when bound by Cu^{2+}; these include Na^{+} and Ca^{2+} channels,[38] GABAA,[39,40] AMPA,[11] and purinergic $P2X_4$ and $P2X_7$ ionotrophic receptors.[8,9] Further investigation is required to elucidate the relative contribution of such a receptor molecule to the synergistic effect of Cu^{2+} on ketamine-induced antinociception at the concentrations used in the present study.

In contrast to the superadditive interaction between copper salt and ketamine in the intraplantar formalin test, this drug combination produced only an additive effect in the orofacial formalin model. This different interaction obtained through isobologram analysis of data could be the result of differences between the spinal and trigeminal nociceptive systems regarding the distribution of neurotransmitter receptors and mechanisms involved. Indeed, although the trigeminal and spinal systems have often been regarded as anatomically and functionally homologous systems, it is known that there are differences. For example, portions of the subnucleus caudalis of the trigeminal spinal nucleus are organized in a different way than the spinal system, and the afferent fibers releasing substance P and calcitonin gene-related peptide show a different pattern of distribution in the trigeminal subnucleus caudalis of adult animals when compared to the spinal dorsal horn.[41,42] In addition to a different organization of the nociceptive afferent input in the trigeminal vs the spinal cord system, the inhibitory projections descending from the rostral ventromedial medulla to the trigeminal subnucleus caudalis and spinal dorsal horn are morphologically and neurochemically distinct.[43] For example, primary afferent neurotransmission to the superficial layers of the trigeminal subnucleus caudalis and spinal dorsal horn is inhibited by different subtypes of the 5-HT1 receptor (the 5-HT1B and 5-HT1D receptor subtypes are effective in the subnucleus caudalis, and the 5-HT1A receptor subtype is effective in the spinal dorsal horn[44]), which may underlie the differential sensitivity to triptans of caudalis neurons compared to spinal dorsal horn neurons.[45] Further, primary trigeminal afferents have a specific endogenous agonist system for the TRPV1 channel—the oxidized linoleic acid metabolites system—that can be activated under conditions of tissue injury.[46] Moreover, glutamate-mediated current density in neurons from the subnucleus caudalis have been reported to be significantly lower than those from cultured spinal dorsal horn neurons,[47] which may have some relation with the inability of Cu^{2+} to potentiate ketamine-induced antinociception upon orofacial testing. However, despite these described differences, the ED50 values exhibited by ketamine and Cu^{2+} during orofacial formalin testing did not differ from those obtained upon intraplantar formalin testing in the present study.

It seems possible that the conclusions arising from the present study on the differential interaction between $CuSO_4$ and ketamine in generating antinociception of formalin-induced nociceptive behavior in territories innervated by trigeminal nerve branches and spinal nerve branches are perhaps only pertinent to chemically induced pain. Indeed, the first phase of the formalin nociceptive response has been proposed to be mediated by activation by formalin of TRPA1 receptor channels expressed in peripheral nociceptors.[48] However, the second phase of the formalin-induced response in rodents has been shown to reflect integration between peripheral (nociceptors) and central (spinal/supraspinal) signaling, including neuronal and glial responses,[49] which are mechanisms relevant to most forms of sustained or chronic pain. Thus, whether there are specific protein targets that are critical for formalin-evoked nociception and their identities, especially for the second phase, are largely unknown. In this regard, it has recently been reported that TRPV4 ion channels are also importantly involved in the trigeminal formalin nociceptive response in addition to TRPA1, the TRPV4 being contributory to all phases of the trigeminal formalin-induced response.[50] Since the mRNA expression for TRPV4, but not for TRPA1, is 10-fold higher in the trigeminal ganglia compared to the dorsal root ganglia,[51] the involvement of TRPV4 may be on the basis of the differential synergy exhibited by $CuSO_4$ and ketamine when tested for antinociception in the orofacial or the intraplantar formalin tests.

Although the mechanism underlying the ability of $CuSO_4$ to exert a synergistic action upon the ketamine antinociceptive effect in spinal-innervated but not trigeminal-innervated territories is still uncertain, this topic could constitute a potential basis for future clinical applications aiming to obtain more antinociception together with a reduction of ketamine side effects, provided the copper/ketamine interaction be proven superadditive in other preclinical models of persistent pain. As pointed out elsewhere,[52] it is not possible to determine synergism in humans due to scientific, practical, and ethical reasons, and thus studies on preclinical drug combinations should be carried out in animals to obtain the basis and rationale for further studies in humans. Ketamine is the most potent NMDA receptor blocker available for clinical use and could be used for postoperative analgesia and for cancer and noncancer chronic pain, but it produces some undesirable dose-related effects, such as psychotomimetic phenomena, that

limit its usefulness.[19,34] On the other hand, copper levels are tightly regulated, and both copper deficit and excess could be deleterious to the organism. The World Health Organization recommends up to 12 µg of dietary copper daily for healthy adult humans,[1] but this higher level could probably be overcome if copper is positioned in the future as an inexpensive analgesic adjuvant or as a drug complex for treating a disease. Studies on in vivo toxicity of copper ions in rats have revealed that high levels of $CuSO_4$ delivered via a gastric tube (60 mg/kg twice a week), but not low levels of salt (10 mg/kg twice a week), induced adverse effects on lipid profiles associated with oxidative stress and diminished activities of antioxidant enzymes, the oral LD_{50} for $CuSO_4$ being 960 mg/kg.[53] In addition, it has been shown that a single oral dose of 500 mg/kg of $CuCl_2$ in the rat increased the brain concentration of Cu^{2+} by about 70%, but in the following 72 hours this level decayed to those of controls.[54] To date, copper has not found many uses in medicine, but further preclinical and clinical studies[55] will most likely lead to some novel applications of copper in the near future. In this regard, very recent data support a likely role of copper ions and copper complexes in diverse areas of medicine, such as angiogenesis,[56] antimicrobial and anticancer drugs,[57–59] cancer biomarkers,[59] treatment of gastrointestinal diseases,[60] and wound healing.[61] Most of these applications are based on the involvement of copper in regulatory, immunologic, and antioxidant functions. In the case of the antinociceptive properties of copper and its ability to synergize the effects of other peripheral and/or central analgesic drugs, additional preclinical and clinical research is required to position copper as an adjuvant analgesic in the future.

Conclusions

Copper salts could be used to synergistically improve the efficacy of some commercial centrally acting analgesic agents, such as ketamine; however, this synergistic action might not have an effect on orofacial pain.

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References

1. World Health Organization. Trace Elements in Human Nutrition and Health. Geneva, Switzerland: World Health Organization, 1996.
2. Turnlund JR, Keyes WR, Peiffer GL, Scott KC. Copper absorption, excretion, and retention by young men consuming low dietary copper determined by using the stable isotope 65Cu. Am J Clin Nutr 1998;67:1219–1225.
3. Stuerenburg HJ. CSF copper concentrations, blood-brain barrier function, and coeruloplasmin synthesis during the treatment of Wilson's disease. J. Neural Transm (Vienna) 2000; 107:321–329.
4. Kardos J, Kovács I, Hajós F, Kálmán M, Simonyi M. Nerve endings from rat brain tissue release copper upon depolarization. A possible role in regulating neuronal excitability. Neurosci Lett 1989;103:139–144.
5. Doreulee N, Yanovsky Y, Haas HL. Suppression of long-term potentiation in hippocampal slices by copper. Hippocampus 1997;7:666–669.
6. Salazar-Weber NL, Smith JP. Copper inhibits NMDA receptor-independent LTP and modulates the paired-pulse ratio after LTP in mouse hippocampal slices. Int J Alzheimers Dis 2011;2011:864753.
7. Kapkaeva MR, Popova OV, Kondratenko RV, et al. Effects of copper on viability and functional properties of hippocampal neurons in vitro. Exp Toxicol Pathol 2017;69:259–264.
8. Coddou C, Villalobos C, González J, Acuña-Castillo C, Loeb B, Huidobro-Toro JP. Formation of carnosine-Cu(II) complexes prevents and reverts the inhibitory action of copper in P2X4 and P2X7 receptors. J Neurochem 2002;80:626–633.
9. Coddou C, Morales B, Huidobro-Toro JP. Neuromodulator role of zinc and copper during prolonged ATP applications to P2X4 purinoceptors. Eur J Pharmacol 2003;472:49–56.
10. Vlachová V, Zemková H, Vyklický L Jr. Copper modulation of NMDA responses in mouse and rat cultured hippocampal neurons. Eur J Neurosci 1996;8:2257–2264.
11. Weiser T, Wienrich M. The effects of copper ions on glutamate receptors in cultured rat cortical neurons. Brain Res 1996; 742:211–218.
12. Horning MS, Trombley PQ. Zinc and copper influence excitability of rat olfactory bulb neurons by multiple mechanisms. J Neurophysiol 2001;86:1652–1660.
13. Aedo F, Delgado R, Wolff D, Vergara C. Copper and zinc as modulators of neuronal excitability in a physiologically significant concentration range. Neurochem Int 2007;50:591–600.
14. Mathie A, Sutton GL, Clarke CE, Veale EL. Zinc and copper: Pharmacological probes and endogenous modulators of neuronal excitability. Pharmacol Ther 2006;111:567–583.
15. Okuyama S, Hashimoto S, Aihara H, Willingham WM, Sorenson JR. Copper complexes of nonsteroidal antiinflammatory agents: Analgesic activity and possible opioid receptor activation. Agents Actions 1987;21:130–144.
16. Tamba BI, Leon MM, Petreus T. Common trace elements alleviate pain in an experimental mouse model. J Neurosci Res 2013;91:554–561.
17. Gumilar F, Agotegaray M, Bras C, Gandini NA, Minetti A, Quinzani O. Anti-nociceptive activity and toxicity evaluation of Cu(II)-fenoprofenate complexes in mice. Eur J Pharmacol 2012;675:32–39.
18. El-Gammal OA, Elmorsy EA, Sherif YE. Evaluation of the anti-inflammatory and analgesic effects of Cu(II) and Zn(II) complexes derived from 2-(naphthalen-1-yloxy)-N'-(1-(pyridine-2-1) ethylidene) acetohydrazide. Spectrochim Acta A Mol Biomol Spectrosc 2014;120:332–339.

19. Tallarida RJ. Drug Synergism and Dose-Effect Data Analysis. Boca Raton: Chapman & Hall, 2000.
20. Tallarida RJ. An overview of drug combination analysis with isobolograms. J Pharmacol Exp Ther 2006;319:1–7.
21. Tawfic QA. A review of the use of ketamine in pain management. J Opioid Manag 2013;9:379–388.
22. Niesters M, Martini C, Dahan A. Ketamine for chronic pain: Risks and benefits. Br J Clin Pharmacol 2014;77:357–367.
23. National Research Council. Guide for the Care and Use of Laboratory Animals, ed 8. Washington D.C.: National Academy, 2011.
24. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 2007;39:175–191.
25. Zhao CS, Tao YX, Tall JM, Donovan DM, Meyer RA, Raja SN. Role of micro-opioid receptors in formalin-induced pain behavior in mice. Exp Neurol 2003;184:839–845.
26. Luccarini P, Childeric A, Gaydier AM, Voisin D, Dallel R. The orofacial formalin test in the mouse: A behavioral model for studying physiology and modulation of trigeminal nociception. J Pain 2006;7:908–914.
27. Desroches J, Bouchard JF, Gendron L, Beaulieu P. Involvement of cannabinoid receptors in peripheral and spinal morphine analgesia. Neuroscience 2014;261:23–42.
28. Katsuyama S, Otowa A, Kamio S, et al. Effect of plantar subcutaneous administration of bergamot essential oil and linalool on formalin-induced nociceptive behavior in mice. Biomed Res 2015;36:47–54.
29. Gonzalez C, Zegpi C, Noriega V, Prieto JC, Miranda HF. Synergism between dexketoprofen and meloxicam in an orofacial formalin test was not modified by opioid antagonists. Pharmacol Rep 2011;63:433–440.
30. Miyamoto M, Tsuboi Y, Honda K, et al. Involvement of AMPA receptor GluR2 and GluR3 trafficking in trigeminal spinal subnucleus caudalis and C1/C2 neurons in acute-facial inflammatory pain. PLoS One 2012;7:e44055.
31. Tallarida RJ. Drug combinations: Tests and analysis with isoboles. Curr Protoc Pharmacol 2016;72:9.19.1–9.19.19.
32. Agotegaray M, Gumilar F, Boeris M, Toso R, Minetti A. Enhanced analgesic properties and reduced ulcerogenic effect of a mononuclear copper(II) complex with fenoprofen in comparison to the parent drug: Promising insights in the treatment of chronic inflammatory diseases. Biomed Res Int 2014;2014:505987.
33. Chizh BA. Low dose ketamine: A therapeutic and research tool to explore N-methyl-D-aspartate (NMDA) receptor-mediated plasticity in pain pathways. J Psychopharmacol 2007;21:259–271.
34. Persson J. Ketamine in pain management. CNS Neurosci Ther 2013;19:396–402.
35. Honey CR, Miljkovic Z, MacDonald JF. Ketamine and phencyclidine cause a voltage-dependent block of responses to L-aspartic acid. Neurosci Lett 1985;61:135–139.
36. MacDonald JF, Bartlett MC, Mody I, et al. Actions of ketamine, phencyclidine and MK-801 on NMDA receptor currents in cultured mouse hippocampal neurones. J Physiol 1991;432: 483–508.
37. Pelissier T, Infante C, Constandil L, Espinosa J, Lapeyra CD, Hernández A. Antinociceptive effect and interaction of uncompetitive and competitive NMDA receptor antagonists upon capsaicin and paw pressure testing in normal and monoarthritic rats. Pain 2008;134:113–127.
38. Popa MO, Lerche H. Cu2+ (1,10 phenanthroline)3 is an open-channel blocker of the human skeletal muscle sodium channel. Br J Pharmacol 2006;147:808–814.
39. Narahashi T, Ma JY, Arakawa O, Reuveny E, Nakahiro M. GABA receptor-channel complex as a target site of mercury, copper, zinc, and lanthanides. Cell Mol Neurobiol 1994;14:599–621.
40. Trombley PQ, Horning MS, Blakemore LJ. Carnosine modulates zinc and copper effects on amino acid receptors and synaptic transmission. Neuroreport 1998;9:3503–3507.
41. Bereiter DA, Hirata H, Hu JW. Trigeminal subnucleus caudalis: Beyond homologies with the spinal dorsal horn. Pain 2000;88:221–224.
42. Ren K, Dubner R. The role of trigeminal interpolaris-caudalis transition zone in persistent orofacial pain. Int Rev Neurobiol 2011;97:207–225.
43. Aicher SA, Hermes SM, Whittier KL, Hegarty DM. Descending projections from the rostral ventromedial medulla (RVM) to trigeminal and spinal dorsal horns are morphologically and neurochemically distinct. J Chem Neuroanat 2012;43:103–111.
44. Connor M. What would 5-HT do? Regional diversity of 5-HT(1) receptor modulation of primary afferent neurotransmission. Br J Pharmacol 2012;167:353–355.
45. Cumberbatch MJ, Hill RG, Hargreaves RJ. Differential effects of the 5HT1B/1D receptor agonist naratriptan on trigeminal versus spinal nociceptive responses. Cephalalgia 1998;18:659–663.
46. Hargreaves KM. Orofacial pain. Pain 2011;152(suppl):s25–s32.
47. Xie YF, Jiang XH, Sessle BJ, Yu XM. Development of regional specificity of spinal and medullary dorsal horn neurons. World J Biol Chem 2016;7:138–145.
48. McNamara CR, Mandel-Brehm J, Bautista DM, et al. TRPA1 mediates formalin-induced pain. Proc Natl Acad Sci U S A 2007;104:13525–13530.
49. Dallel R, Raboisson P, Clavelou P, Saade M, Woda A. Evidence for a peripheral origin of the tonic nociceptive response to subcutaneous formalin. Pain 1995;61:11–16.
50. Chen Y, Kanju P, Fang Q, et al. TRPV4 is necessary for trigeminal irritant pain and functions as a cellular formalin receptor. Pain 2014;155:2662–2672.
51. Vandewauw I, Owsianik G, Voets T. Systematic and quantitative mRNA expression analysis of TRP channel genes at the single trigeminal and dorsal root ganglion level in mouse. BMC Neurosci 2013;14:21.
52. Chou TC. Preclinical versus clinical drug combination studies. Leuk Lymphoma 2008;49:2059–2080.
53. Galhardi CM, Diniz YS, Faine LA, et al. Toxicity of copper intake: Lipid profile, oxidative stress and susceptibility to renal dysfunction. Food Chem Toxicol 2004;42:2053–2060.
54. Lee IC, Ko JW, Park SH, et al. Comparative toxicity and biodistribution of copper nanoparticles and cupric ions in rats. Int J Nanomedicine 2016;11:2883–2900.
55. Szymański P, Frączek T, Markowicz M, Mikiciuk-Olasik E. Development of copper based drugs, radiopharmaceuticals and medical materials. Biometals 2012;25:1089–1112.
56. Saghiri MA, Asatourian A, Orangi J, Sorenson CM, Sheibani N. Functional role of inorganic trace elements in angiogenesis—Part II: Cr, Si, Zn, Cu, and S. Crit Rev Oncol Hematol 2015;96:143–155.
57. Patil SA, Patil SA, Patil R, et al. N-heterocyclic carbene metal complexes as bio-organometallic antimicrobial and anticancer drugs. Future Med Chem 2015;7:1305–1333.
58. Kalinowski DS, Stefani C, Toyokuni S, et al. Redox cycling metals: Pedaling their roles in metabolism and their use in the development of novel therapeutics. Biochim Biophys Acta 2016; 1863:727–748.
59. Zhou M, Tian M, Li C. Copper-based nanomaterials for cancer imaging and therapy. Bioconjug Chem 2016;27:1188–1199.
60. Siczek K, Zatorski H, Fichna J. Silver and other metals in the treatment of gastrointestinal diseases. Curr Med Chem 2015; 22:3695–3706.
61. Kornblatt AP, Nicoletti VG, Travaglia A. The neglected role of copper ions in wound healing. J Inorg Biochem 2016;161:1–8.