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The Effect of Copper Chloride (CuCl2) on Pain and Inflammatory Paw Edema in Rats

H. Nikbakhty (MSc)[1], M. Fereidoni (PhD)*[1], A. Asadollahi (PhD)[1]

1.Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, I.R.Iran

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ABSTRACT

BACKGROUND AND OBJECTIVE: As a crucial micronutrient, copper ion is engaged in various biochemical pathways and affects central nervous system, pain, and inflammation. Considering the significance of pain in physical and mental condition of patients, the present study aims to find new ways to reduce pain by investigating the effect of copper chloride on thermal and chemical pain and inflammatory edema through central and peripheral administrations.

METHODS: In this empirical study, 77 male Wister rats (200–250g) were divided into 11 groups (n=7) including control group (with no treatment), sham 1 (saline, i.p) receiving 5, 10, 20 and 100 mg/kg $CuCl_2$ intraperitoneally (i.p), sham 2 (saline, intrathecal (i.t)) receiving 0.002mg/10µl and 0.02mg/10µl $CuCl_2$ intrathecally (i.t), sham 3 group receiving saline plus naloxone intraperitoneally (i.p) and the group receiving 10 mg/kg $CuCl_2$ plus 2 mg/kg naloxone intraperitoneally (i.p). Thermal and chemical pain and the volume of inflammatory edema were assessed using tail flick, formalin and plethysmometery tests, respectively. Elevated plus maze and rotarod tests were used to examine the side effects of $CuCl_2$.

FINDINGS: 10 and 20 mg/kg $CuCl_2$ (i.p) respectively reduced thermal pain (1.69±0.38 and 1.55±0.53) ($p<0.001$) and chemical pain ($p<0.01$) and reduced inflammatory paw edema (70.43±20.96 and 70.38±29.01) ($p<0.01$). However, rats receiving 100 mg/kg $CuCl_2$ did not survive. On the other hand, naloxone abolished the analgesic effect of CuCl2 (0.015±0.055) ($p<0.001$). Intrathecal administration of 0.002mg/10µl $CuCl_2$ had no significant effect on pain but 0.02 mg/1 0 µl $CuCl_2$ reduced chemical pain ($p<0.01$). $CuCl_2$ had no effect on balance and anxiety.

CONCLUSION: Since administration of naloxone as opioid receptor antagonist abolished the analgesic effect of $CuCl_2$, $CuCl_2$ may induce analgesia by increasing the sensitivity of opioid receptors to endogenous opioids.

KEY WORDS: *$CuCl_2$, Thermal pain, Chemical pain, Inflammatory edema*.

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***Corresponding author: M. Fereidoni (PhD)**
Address: Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, I.R.Iran
Tel: +98 51 38762227
E-mail: fereidoni@um.ac.ir

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Introduction

Pain is a complicated sensory perception and inadequate control of pain has adverse effects on physiological and psychological condition of patients (1). One the best ways to relieve pain is through natural substances such as micronutrients. Copper ion is one of the vital micronutrients of body and is involved in various reactions and biochemical pathways (2). The activity of catalase and glutathione peroxidase enzymes depends on copper ion. On the other hand, copper ion is important for development and maintenance of body's immune system and acts as a cofactor for superoxide dismutase enzyme.

It is also important for antioxidant defense and eradication of free radicals. Studies indicate that copper ion has anti-inflammatory and analgesic properties. These results show that combination of copper ion with nonsteroidal anti-inflammatory drugs enhances their therapeutic properties (3); combination of copper ion with these drugs imitates the activity of superoxide dismutase enzymes and enhances the ability of digesting free radicals (4).

It is also demonstrated that combination of copper ion with fenoprofen and imidazole has greater analgesic effect on inflammatory pain and suppression of visceral pain (5). Moreover, the visceral analgesic effects and arthritis were reinforced by various combinations of copper (6). Some studies suggest the use of metallic tools such as copper bracelets to relieve joint pain. It is generally believed that copper is absorbed through the skin and affects the copper-dependent enzymes and decreases inflammatory pain (7). According to another study, using a combination of copper ion with salicylic for rats with arthritis reinforced the analgesic effects and these analgesic effects can be independent of the presence of inflammation or analgesic effect of inflammation reduction (8).

Moreover, some studies indicate increased sensitivity of opioid receptors in the presence of copper ion. Intracerebral administration of copper ion to rats demonstrated analgesic effects reversible with naloxone. Thus, copper ion probably occupies the binding site of opioid receptor (6). Considering the importance of pain and man's efforts throughout history for finding ways to confront pain and reduce it and due to several results indicating the role of copper ion in reinforcing the analgesic and anti-inflammatory effects of nonsteroidal anti-inflammatory drugs, the present study was conducted to investigate the effect of intraperitoneal and intrathecal administration of copper chloride ($CuCl_2$) on thermal pain, chemical pain due to plantar injection of formalin and inflammatory edema induced by formalin in rat paw. The side effects of $CuCl_2$ on anxiety and motor coordination were also examined using elevated plus maze and rotarod tests.

Methods

In this empirical study, 77 male Wister rats (200–250g) were used. Rats were kept in an animal house in Department of Biology, Ferdowsi University of Mashhad under standard conditions with 12/12 light cycle, 50% humidity at 21ºC and free access to food and water. Rats were divided into 11 groups (n=7) including control group (with no treatment), sham 1 (saline, i.p), groups receiving 5, 10, 20 and 100 mg/kg $CuCl_2$ intraperitoneally (i.p), sham 2 (saline, intrathecal (i.t)), groups receiving 0.002mg/10µl and 0.02mg/10µl $CuCl_2$ intrathecally (i.t), sham 3 group receiving saline plus naloxone 2 mg/kg intraperitoneally and the group receiving 10 mg/kg $CuCl_2$ plus 2 mg/kg naloxone intraperitoneally.

Thermal and chemical pain and the volume of inflammatory edema were assessed using tail flick, formalin and plethysmometery tests, respectively. Elevated plus maze and rotarod tests were used to study the side effects of $CuCl_2$. All experiments on laboratory animals were conducted based on the ethics of The International Committee for Laboratory Animal Science (9).

In this research, copper chloride was obtained from Fluka Company in New Zealand and all doses were extracted according to the study of Okuyama et al. (6). In the groups receiving $CuCl_2$ with naloxone 20 minutes after intraperitoneal administration of $CuCl_2$, 2 mg/kg naloxone was administered intraperitoneally and then the rats were examined in term of pain and inflammation.

Intrathecal (i.t) administration: The rats were anesthetized through intraperitoneal injection of ketamine (100 mg/kg) and xylazine (200 mg/kg) to perform surgery and cannulation in the spinal cord for spinal administrations. Then, their head was fixed using a stereotaxic device and the neck muscles were set aside with a small incision. Then, a small incision was created in the middle of Atlanto-occipital membrane Atlas and the cerebrospinal fluid was drained. Then, a PE-10 (with 11 cm length) was inserted slowly in subarachnoid space towards the

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lumbar part of spinal cord in a way that 8 cm was placed in subarachnoid space and 3 cm was placed outside from the vertebral column for the sake of administrations. We should not observe any kind of defect in movement of animals after cannulation. One week after recovery, the drug was intrathecally injected in the desired volume through cannula (10). To administer 10 mg/kg and 100 mg/kg intrathecally, it was necessary to modify the desired concentration of each dosage during peritoneal administration. Therefore, considering that the lumbar spinal cord in rats weighs around 0.2 g and the permitted administration volume in spinal cord of rats is 10 µl, the concentration of desired dosage was calculated.

Tail flick test: Tail flick test was used to measure thermal pain threshold. In this test, an intense thermal stimulant at about 40 °C was applied to the middle third of the animal's tail and the interval between the beginning of heating until movement of the tail by the animal was assessed. Thermal pain threshold in rats was determined to be 30 minutes after intraperitoneal administration and 5 minutes after intrathecal administration (11). The level of change in thermal pain threshold was obtained through calculation of the difference in the average delay time, before and after treatment.

Formalin test: Formalin test was used to evaluate the analgesic effects of substances on sustained chemical pain, during which tissue damage occurs. Response to pain in this test consists of two stages. In the first stage, the animal feels pain for a short period and then the pain is relived for a few minutes and in the second stage, the animal feels pain for a longer period. In this test, 0.05 ml formalin 2.5% was injected subcutaneously into the plantar region of a back paw. The formalin test was run 30 minutes after intraperitoneal administration and 5 minutes after intrathecal administration. Then, the animal's pain behavior was evaluated for one hour. In order to quantify the intensity of pain and presenting a better comparison between data on animal's behavior regarding formalin induced pain, a scoring system was used as follows: 0 in case of feeling no pain, 1 in cases where the rats placed their injected paws on the ground, 2 in cases where the rats kept their paws on the air all the time and 3 in cases where the rats moved, bit or licked their paws (11).

Plethysmometery test: Plethysmometery test is the most common type of inflammation assessment, which calculates the inflammatory edema volume of paw. In this test, 0.05 ml formalin 2.5% was injected into animal's paw and the volume of paw was determined before plantar injection of formalin and one hour after that. Animal's paw was marked at ankle area and was inserted in liquid column placed on an accurate digital balance, up to the marked area. Finally, the level of inflammatory edema was detected using V_t–V_0 relationship (V_0 is the initial volume of paw and V_t is the size of paw one hour after plantar formalin injection) [Volume is calculated using the equation $V=W/\rho$ (V is standing for the volume of paw, W for the weight of liquid which is determined by digital balance and ρ for specific weight of liquid)] (12).

Elevated plus maze test and rotarod test: Elevated plus maze test is used to determine anxiety. To run this test, each rat is located at the center of elevated plus maze. The number of entries into an arm, and the time spent in the open and closed arms are recorded for five minutes. The longer a rat stays in a closed arm, the animal suffers from more anxiety (13). Rotarod test is used to assess the ability to maintain motor coordination. Under each rod, there was a pedal and as the animal fell on the pedal, the timer stopped and the speed and duration of maintaining coordination on the rotating rod was recorded (14).

Ultimately, the data were presented in the form of mean±SEM and GraphPad Prism 5 was used to analyze the acquired data. One-way ANOVA statistical test was used in tail flick test, plethysmometery test, elevated plus maze test and rotarod test to evaluate the effectiveness of the treatment and T-test (Newman-Keuls) was used to compare the mean data between groups.

To analyze the data in formalin test, repeated measure ANOVA statistical test was separately used in each treatment to evaluate the effect of time on changes in pain and after specifying the effect of time, One-way ANOVA statistical test was used to evaluate the effect of treatments at each moment including 5, 10,… minutes after plantar formalin injection and if the result was significant, T-test (Newman-Keuls) was used and $p<0.05$ was considered significant.

Results

Since there was no significant difference between sham 1, 2 and 3 and control group in different tests, sham 1 group was only used in diagrams for comparing with other treatments. Results of Tail flick test demonstrated that intraperitoneal administration of

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dose-dependent 5, 10 and 20 mg/kg $CuCl_2$ increased thermal pain threshold and caused analgesia. Comparing the most effective dosage of $CuCl_2$ in intraperitoneal administration (10 mg/kg) and its equivalent concentration (0.002 mg/10 µl) and equivalent concentration of 100 mg/kg (0.02 mg/10 µl) in intrathecal administration demonstrated that intrathecal administration of $CuCl_2$ does not elevate the pain threshold and does not reduce pain as intrathecal administration of $CuCl_2$ does not cause a significant change in thermal pain threshold respect to the sham. Moreover, the most effective dosage of intraperitoneal administration of $CuCl_2$ (10 mg/kg) with opioid system antagonist (2 mg/kg naloxone) was used to study the possible relationship between thermal analgesia due to intraperitoneal administration of $CuCl_2$ and opioid system. Results of the study demonstrated that the effects of $CuCl_2$ on thermal pain threshold and analgesia were nullified by naloxone (Fig 1).

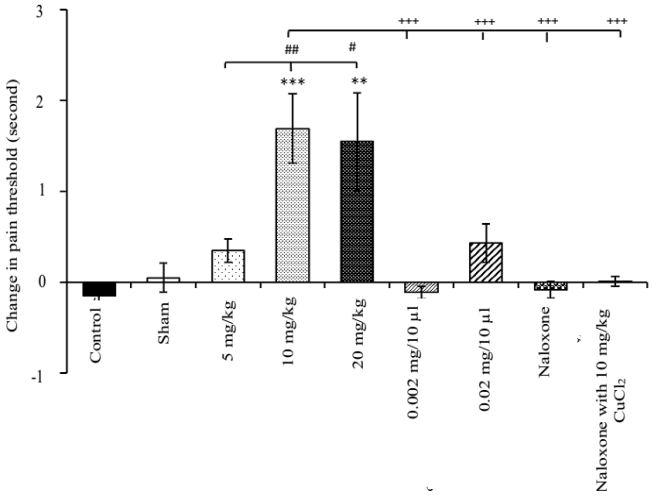

Figure 1. Tail flick test results: The effect of intraperitoneal and intrathecal administration of $CuCl_2$ on thermal pain threshold in different studied groups

**p<0.01 and ***p<0.001 compared with control group, #p<0.05 and ##<0.01 compared with the group receiving 5 mg/kg $CuCl_2$ and +++p<0.001 compared with the group receiving 10 mg/kg $CuCl_2$. The data were illustrated as mean±SEM (n=7).

In formalin test, 10 and 20 mg/kg intraperitoneal administration of $CuCl_2$ significantly reduced chemical pain compared with control group. However, 5 mg/kg intraperitoneal administration of $CuCl_2$ did not cause a significant change compared with control group (Fig 2–A). Comparing the most effective dosage of $CuCl_2$ in intraperitoneal administration (10 mg/kg) and its equivalent concentration (0.002 mg/10 µl) and equivalent concentration of 100 mg/kg (0.02 mg/10 µl) in intrathecal administration demonstrated that 0.002 mg/10 µl intrathecal administration had no significant effect on intensity of chemical pain, while 0. 02 mg/10 µl intrathecal administration was relatively effective on chemical pain reduction (Fig 2–B).

Therefore, based on the mentioned results both for the thermal and chemical pain evaluations, it is possible that peripheral mechanisms are more involved with the analgesic effects of $CuCl_2$. The most effective dosage of $CuCl_2$ in intraperitoneal administration (10 mg/kg) and 2 mg/kg naloxone were used to study the relationship between analgesia due to $CuCl_2$ administration and opioid system. There was no significant difference between groups of sham, 2 mg/kg naloxone and 10 mg/kg $CuCl_2$ with naloxone, while administration of 10 mg/kg $CuCl_2$ alone caused analgesia significantly (Fig 2–C).

On the other hand, plethysmometery test indicated that intraperitoneal administration of 10 and 20 mg/kg $CuCl_2$ significantly decreased inflammatory edema due to plantar formalin injection compared with control group, while 5 mg/kg dosage could not decrease inflammatory edema volume significantly. To study the central and peripheral effects of $CuCl_2$ administration, plethysmometery test was run between the most effective dosage of $CuCl_2$ in intraperitoneal administration (10 mg/kg) and its equivalent concentration (0.002 mg/10 µl) and equivalent concentration of $CuCl_2$ lethal dose (100 mg/kg), 0.02 mg/10 µl in intrathecal administration and demonstrated that contrary to intraperitoneal administration, intrathecal administration of $CuCl_2$ at the most effective dosage does not have a significant effect on inflammatory edema volume. Moreover, to study the mechanism of $CuCl_2$ on inflammatory edema, the effective dosage of 10 mg/kg $CuCl_2$ along with opioid antagonist (2 mg/kg naloxone) was used in intraperitoneal administration.

Results of the study demonstrated that administration of naloxone alone or with the most effective dosage of $CuCl_2$ did not create a significant change in inflammatory edema volume of paw, while intraperitoneal administration of 10 mg/kg $CuCl_2$ alone decreased inflammatory edema volume of paw (Fig 3). Elevated plus maze and rotarod tests were used to study the side effects of $CuCl_2$. According to elevated plus maze test results, intraperitoneal administration of 10 mg/kg $CuCl_2$ did not create anxiety or movement disorders in the animals (Fig 4).

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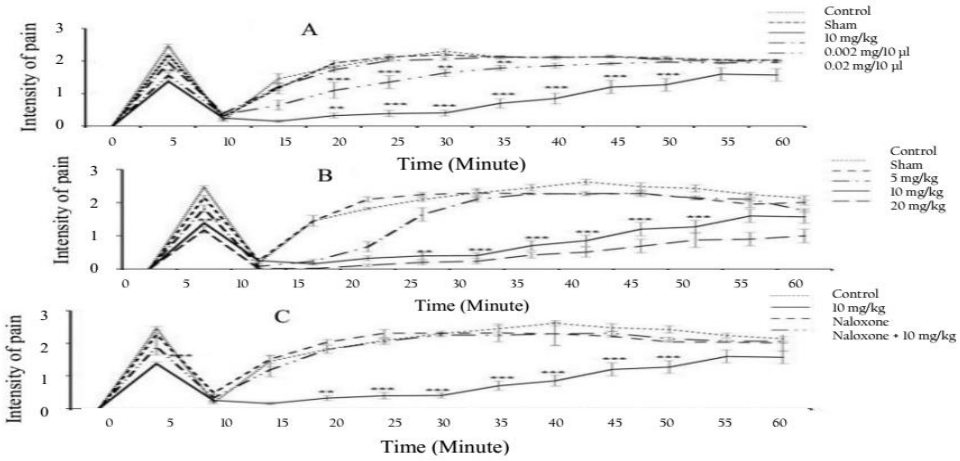

Figure 2. Formalin test results: The effect of intraperitoneal and intrathecal administration of $CuCl_2$ on intensity of chemical pain in different studied groups.

A. intraperitoneal administration of 10 mg/kg $CuCl_2$ decreased chemical pain. B. only intrathecal administration of 100 mg/kg $CuCl_2$ relatively decreased chemical pain. C. administration of naloxone along with the most effective dosage of $CuCl_2$ nullified the analgesic effects of $CuCl_2$. (**p<0.01 and ***p<0.001 compared with control group). The data were illustrated as mean±SEM (n=7).

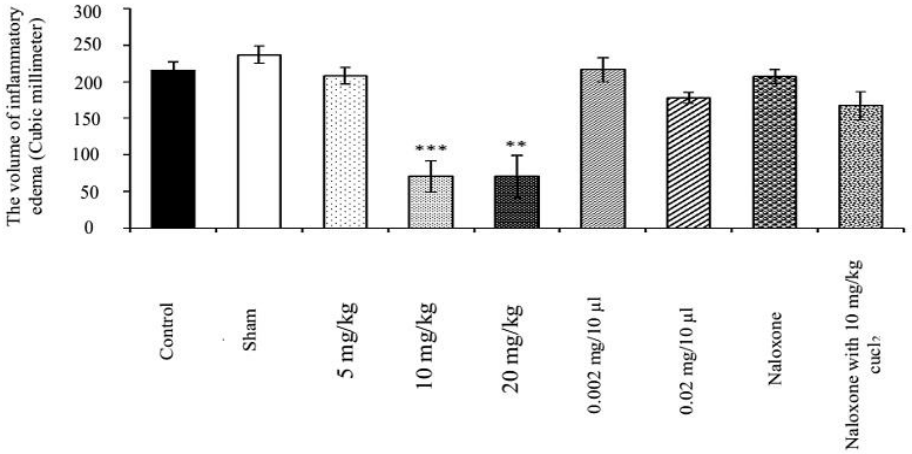

Figure 3. Plethysmometery test results: The effects of intraperitoneal and intrathecal administration of $CuCl_2$ (both the concentrations of 0.02 and 0.002 mg/10 µl) on inflammatory edema volume of paw in the studied groups. Only intraperitoneal doses of 10 and 20 mg/kg reduced formalin induced paw edema significantly.

**p<0.01, ***p<0.001 compared with control group. The data were illustrated as mean±SEM (n=7).

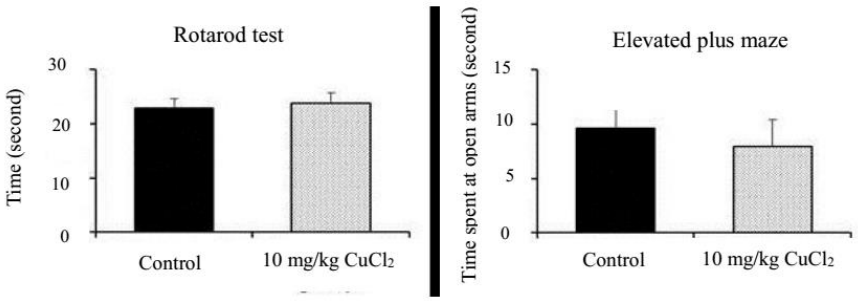

Figure 4. Elevated plus maze and rotarod test results: The comparison between anxiety in elevated plus maze test and motor coordination in rotarod test in control group and intraperitoneal administration of the most effective dosage of $CuCl_2$ did not reveal a significant difference. The data were illustrated as mean±SEM (n=7).

Discussion

Results of the study demonstrated that intraperitoneal administration of $CuCl_2$, particularly at the most effective dosages (10 and 20 mg/kg), increased thermal pain threshold and decreased chemical pain and inflammatory edema volume significantly. These effects were nullified with co-administration of naloxone and the most effective

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dosage of $CuCl_2$. Intraperitoneal administration of 100 mg/kg $CuCl_2$ led to the death of rats caused by copper ion poisoning. Peripheral poisoning caused by copper ions ultimately leads to death of cell probably by creating intracellular free radical and changing the cell membrane through lipid peroxidation processes and changing the shape of proteins (15).

Intrathecal administration of the most effective equivalent concentration of $CuCl_2$ (0.002 mg/10 µl) did not show a significant effect on reduction of thermal and chemical pain and the volume of inflammatory edema. However, intrathecal administration of equivalent concentration of $CuCl_2$ lethal dose (0.02 mg/10 µl) created a significant decline in the intensity of chemical pain despite creating signs of poisoning in intraperitoneal administration. Tacking together, $CuCl_2$ at spinal cord level probably affects opioid receptors may be by a dose dependent manner, although the concentration range used here is toxic and is not advised for therapeutic applications. Previous studies specified that the sensitivity of opioid receptors could be increased in the presence of copper ion and intracerebral administration of cupric ion (Cu2+) causes analgesic effects reversible with naloxone (6, 16). Moreover, intraperitoneal administration of copper sulfate reinforced the analgesic effect of morphine. Copper sulfate alone have been able to increase mechanical pain threshold (17).

Moreover, studies demonstrated that treatment with a thiol such as Dithiothreitol nullified the analgesic effects of copper ion. Thus, copper ion probably makes opioid receptors more sensitive to endogenous opioids through oxide-resuscitation mechanisms and causes analgesia (16). Therefore, considering the results of the present study and reversibility of analgesic effects of copper ions by naloxone, it is possible that copper ion increases the sensitivity of opioid receptors to endogenous opioids in the brain, environment or both, but does not possibly affect pain pathways within the spinal cord and in this way reinforces the analgesic effects of opioid system in the brain and environment and reveals analgesic effects. Previous studies demonstrated that combination of copper ion with NSAIDs drugs reinforce the analgesic and anti-inflammatory effects of these drugs (3). In this regard, combination of copper ion with fenoprofen can significantly decrease the volume of inflammatory edema induced in rat paw compared with fenoprofen alone (4). Anti-inflammatory effects of copper ions were also demonstrated in this study. Moreover, results indicated that during peripheral damage to tissue, involved cells in immune system secrete prostaglandin and interleukin (18). However, copper ion inhibits prostaglandin E2. In addition, by inhibiting the calcium channel, copper ion significantly inhibits the intracellular replay of calcium and interferes with histamine release. These events are directly related to anti-inflammatory actions of this cation (19). Since a great deal of pain in the second stage of formalin test is related to peripheral inflammatory events (20), it is possible that administration of $CuCl_2$ significantly decreases chemical pain in this stage through aforementioned mechanisms.

On the other hand, previous studies demonstrated that pretreatment with opioids inhibits chemical activity of monocytes and neutrophils and the subsequent inflammatory processes (21). Moreover, administration of morphine effectively inhibits the migration of microglial cells in a dose-dependent manner (21). Therefore, it can be suggested that as sensitivity of opioid receptors to endogenous opioids increases, copper ion decreases inflammatory processes and inflammatory edema induced by formalin. Overall, according to the results of the present, since administration of naloxone nullifies the analgesic effects of $CuCl_2$ as opioid receptor antagonist, $CuCl_2$ probably decreases thermal and chemical pain and the volume of inflammatory edema through increasing the sensitivity of opioid receptors primarily at the peripheral level and less at the central level.

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