Heavy Metal Trace Elements Induced Antinociception in an Experimental Mouse Model

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Heavy metal trace elements (HMTE) represent a group of metals or metalloids, present in minute amounts in humans but also frequently present in various dietary supplements on the market, including those for pain therapy. Cobalt (Co), Nickel (Ni) or Molybdenum (Mo) are mostly associated with toxic effects. Previous in vitro studies on heavy metals and nociception and their potential influence on pain treatment have yielded limited results, with very few in vivo data available. We hypothesized that Co, Ni and Mo have direct in vivo antinociceptive effects. Swiss mice were intraperitoneally (i.p) injected with incremental doses of Co and Ni chlorides, sodium Mo or saline and evaluated by hot plate (HP) and tail flick (TF) tests for central antinociceptive effect, writhing test (WT) for visceral antinociceptive effect and activity cage (AC) test for spontaneous behaviour. Co induced a dose dependent pain inhibition in HP/TF tests (30.87%/16.64%) and WT (100%/ 77.23%). Ni and Mo salts showed dose dependent anti-nociceptive effects for HP/TF (20.41%/13.4%) and WT (67.41%/67.86%). Only Co and Mo salts have displayed significant effects in AC (Co anxiolytic/depressant; Mo sedative). All tested metals induced various degrees of anti-nociceptive effects, especially in visceral pain tests, supporting HMTE as an important nociception investigation target. More importantly our results make a step forward in the investigation of how these trace elements may influence the traditional drug therapy in patients experiencing pain, when administered as daily food intake or dietary supplements.

Keywords: nickel; molybdenum; cobalt; pain; dietary supplements.

Heavy metal trace elements (HMTE) represent a group of metals or metalloids present in minute amounts in humans and include Cobalt (Co), Nickel (Ni) and Molybdenum (Mo), which are considered essential for human health [1].

The rationale of our study is based on several critical statistical data:

- Over 50.000 dietary or food supplements are on the market today with many containing Co, Ni and Mo.

- 40% of the US general population [2] and more than 65% of chronically ill patients take nutritional supplements [3, 4].

- Pain, and especially chronic pain, affects more than 1.5 billion people worldwide.

- The inclusion of so called pain food supplements has become a common therapeutic approach.

These data intrigued and challenged the authors to try to investigate if there are any connections between these HMTE and pain reception, transmission and modulation.

We believe that, given the enormous quantities of food supplements consumed in the world (including HMTE), with more than 1 billion people with pain worldwide, the scarcity of scientific information of the matter, and the lack of any guidelines or regulations for the healthcare provider or the public for that matter, it is highly important and urgent for both the scientific community as well as the general public to investigate, understand and take eventual measures regarding the HMTE in food supplements in patients with pain.

For years, Co, Ni or Mo were mostly associated with toxic effects and scarcely investigated for other physiological roles, especially in pain generation, transmission or modulation.

Co is a constituent of cobalamin, and this was considered its only role. Dietary intake varies between 5 - 50µg/day. Co is a potent inducer of oxidative stress leading to toxicity, carcinogenicity, DNA damage and sister-chromatid exchange [5]. Co shows cytotoxic effects on various cell types including neurons [6], can induce apoptosis and necrosis [7] but also damage mitochondrias [8]. Co cations can also block Ca-mediated neural transmission, following central administration.

No direct investigation has been performed so far regarding its role in pain following peripheral administration.

Ni plays an important role in animals [9] while low levels are reducing growth and severely interfere lipid metabolic pathways in rats [10]. Dietary intake can reach 1 mg/day [11]. Ni role in nociception has been little investigated. Ni is a non-selective Ca channels blocker, inhibiting the delivery

of pain mediators [12, 13]. Ni may induce direct contact dermatitis. Pain and itching sensory neurons are part of the DRG and trigeminal system and Ni effect in itching induction can be amplified by tissue acidosis [14]. Chronic exposure to Ni can lead to impaired olfaction and anosmia [15], neurotoxicity, headaches, lethargy and ataxia. Ni exposure damages the mitochondrial function and impairs cell viability, inhibits the cell proliferation, blocks neuronal Ca channels, including voltage-dependent T-type and R-type [16, 17], ASIC's [18] as well as GABA-activated channels [19].

Mo, part of several redox enzymes, is involved in the control mechanisms of purines and fats metabolism. Dietary intake ranges from 0.16–0.2 mg/day [20]. Data on Mo and pain is also very limited. A single pilot clinical study [21] showed a significant pain relieve in the Mo treated group.

Modern pain treatments are frequently associated in patients with dietary supplements containing Co, Ni or Mo. These HTME's participation in pain remains a largely overlooked and under investigated subject, especially for in-vivo settings.

Our purpose was to evaluate the in-vivo effects on nociception of these three HMTE's in two different doses, in a murine model.

Experiemntal part
Materials and methods

Various groups of 8 mice each were selected and specific formulations were injected intraperitoneally. Control groups received 0.1mL of 0.9% saline while test groups received Co chloride (doses of 3.75 and 7.5 mg/kg b.w respectively), Ni chloride (doses of 0.5 and 2 mg/kg b.w. respectively) and Na-molybdate (doses of 25 and 50 mg/kg b.w respectively). All reagents had analytical purity, being purchased from Sigma Aldrich Chemie (GmbH USA).

All calculations were made on the chemicals used (not metal component alone).

The doses administered were calculated as fractions of known, published intraperitoneal IP LD50 in mice. We did not use ppm data, as standard toxicity tests and data use LD50 and not ppm.

Our experimental models used doses 5 to 96 times less that known LD50's and did not induce any significant toxic effect. The used doses were:
- 1/24 and 1/12 for Cobalt chloride of LD50. (LD50 90mg/kg b.w.) [22].
- 1/96 and 1/24 for Nickel Chloride (LD50 48 mg/kg b.w.) [23].
- And 1/10 and 1/5 for Sodium Molybdate (LD50 257 mg/kg b.w.) [24].

The pH of the solutions was slightly acid but still at physiological levels. The solvent of the chemical compound was saline (pH 5.5) [25], which is also physiological for IP administration and had no effects in control animals. The trace elements however, did not influence significantly the pH of the injected solutions. Thus, there was no need to use a buffer to attenuate for possible acidity, if any.

Experimental procedures used in the present study were developed in respect to European Ethic Committee regulations, approved and applied by local academic Animal Care and Use Committee. Male Swiss mice, weighting 35 g ± 2 g were provided by the animalery in Central Drug Testing Lab of the „Gr. T. Popa" University of Medicine and Pharmacy Iasi, Romania. All animals were hosted in a temperature controlled environment (21 ± 2°C)

with a 12 h/12 hlight/dark cycle. Each cage included only 4 mice that were allowed to acclimate for 1 day prior use, using full access to food and water [26, 27].

Nociception studies

Thermally induced pain is commonly evaluated by HP (hot plate) and TF (tail flick) assays. Both methods were used to assay the antinociceptive effects of the salts mentioned in materials and methods. TF test supposes animal placement in restraining cages for at least 5 min prior to measurement procedure. The dorsum of the lower third on mice tail was exposed to a constant intensity heat; while the animal flicked the tail as a response to the thermal signal, the exposure was arrested automatically. TF latency was extended no longer than 15 s to minimize or avoid tissue damages. All assays supposed measurements at 15', 30', 45' and 1h respectively following exposure to the assay salts or controls.

HP assay was developed in mice, using the Ugo Basile HP device. While temperature ranged around 54 ± 0.5°C, latencies were evaluated by measuring time from mice placement on a heated surface until the first reactions to nociception appeared. These reactions may be included in following groups (i) hind paw licking, (ii) vocalization, or (iii) escape response. Measurements to detect analgesic response were performed at onset and at 15', 30', 45' and 1h following salts or saline control intraperitoneal administration. The HP test required a cut-off time of 30 s. Only products that induced a significant increase in the nociceptive thresholds were considered to be antinociceptive [27].

An important checkpoint while evaluating antinociceptive effect on visceral pain is represented by a peripheral mechanism assay (writhing test to acetic acid). This assay is developed by intraperitoneal injection of 0.1 mL of 1% acetic acid while recording abdominal stretching in manually restrained mice. In the first seconds following acetic acid injection, mice are being placed in a large glass cylinder. Mice abdominal stretches were counted for a 30 min interval in successive 5 min intervals, starting at 5 min from intraperitoneal injection. Evaluated salts were injected at 5 minutes before acetic acid administration. For each group of 8 mice, mean values were reported (±S.E.M.). Antinociceptive response was considered for the treatments that induced a significant decrease in the number of abdominal stretches. Prior mice sacrifice, their biological evolution was followed for another 72 h.

Unprompted behaviour required Activity Test Cage evaluation.

This assay records spontaneous coordinate activity and its variations in mice. All weight measurements and assay procedures were performed between 9.00 - 11.00 AM. An Ugo Basile Activity Cage System was used to record horizontal and vertical activity, as the total count for beam interruptions for a 2 min observation time. Evaluated products were administered 15 min prior this test. Spontaneous behaviour test was performed in blind (the experimenter was not aware of the treatment received by the animals). The activity cage test was a complementary evaluation, which sought to investigate if, there are, any spontaneous behavioral changes in the test animals for a better interpretation of the pain evaluation tests. The conclusion on the sedative or anxiolytic effects was based on well-established literature data using the activity cage and spontaneous behavior testing.
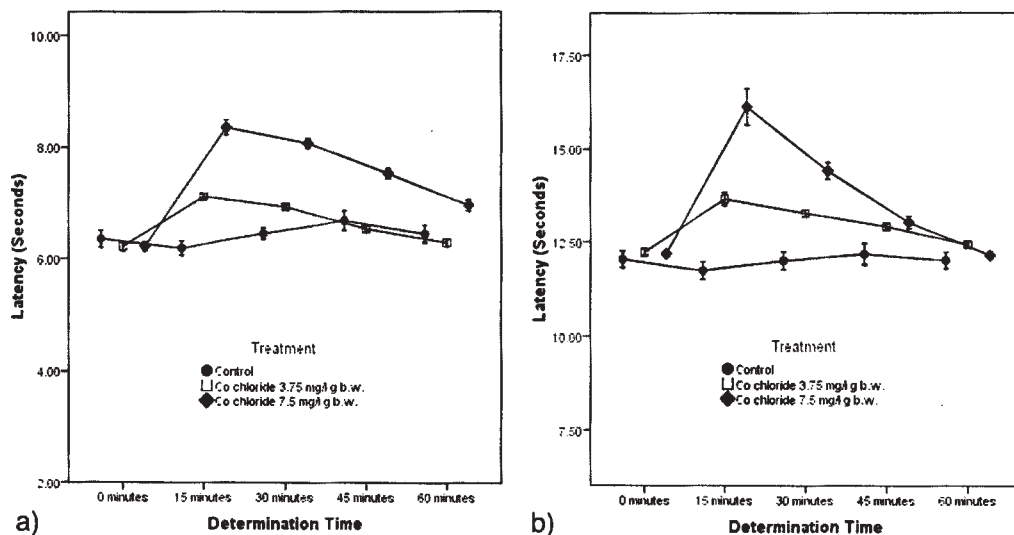
Statistics

Fig. 1. a. Latencies for TF test, after intraperitoneal administration of Co chloride in different doses (3.75 and 7.5 mg/kg respectively), b. Latencies for HP test, following Co chloride intraperitoneal injection in different doses (3.75 and 7.5 mg/kg respectively)

Table 1
AVERAGE PAIN INHIBITION VALUES FOR TESTED SUBSTANCES IN TF AND HP ASSAYS FOLLOWING ADMINISTRATION Co CHLORIDE 3.75 AND 7.5 mg/kg, Ni CHLORIDE 0.5 AND 2.0 mg/kg, SODIUM MOLYBDATE 25 AND 50 mg/kg

Drug-test/time interval	0'	15'	30'	45'	60'
Co chloride 3.75 TF	0	10.22	8.10	3.55	0.79
Co chloride 7.5 TF	0	24.31	21.12	14.96	8.41
Co chloride 3.75 HP	0	8.03	5.86	3.85	1.12
Co chloride 7.5 HP	0	22.13	12.52	4.74	-0.25
Ni chloride 0.5 TF	0	9.70	6.94	4.36	0.13
Ni chloride 2 TF	0	13.41	20.42	13.40	9.57
Ni chloride 0.5 HP	0	3.37	1.68	0.48	-0.73
Ni chloride 2 HP	0	11.32	19.01	11.70	6.91
Sodium Molybdate 25 TF	0	8.79	6.87	3.18	-0.50
Sodium Molybdate 50 TF	0	12.42	10.31	6.59	1.58
Sodium Molybdate 25 HP	0	4.08	3.04	1.68	-0.25
Sodium Molybdate 50 HP	0	9.22	7.95	3.97	1.03

Statistic evaluation was performed by an SPSS 16.0 for windows software package. ANOVA one-way method and Bonferro post-hoc tests were used to express differences between treatment groups. The p values less than 0.05 were used to indicate a significant difference for all tests.

Pain inhibition to various stimuli [28] was also calculated according to formula:

$$\% \text{ Inhibition} = [(T_x - T_0)/(T_m - T_0)] \times 100$$

where T_0 represents the latency prior drug administration (onset), T_x represents the latency for different consecutive time intervals and T_m is the cut-off time (maximum time allowed) to prevent tissue lesions in mice tail. T_m is the maximum time allowed (cut-off time) to avoid any possible lesions to the test animal.

Results and discussions
Results obtained following TF test showed that Co chloride is able to extend TF latency at 15, 30, 45 and 60 min from intraperitoneal administration, thus indicating a significant antinociceptive effect. This effect seems to be dose-dependent as TF latency increases. At a dose of 3.75 mg/kg Co chloride, the TF latency is slightly increased at 15 and 30 min, with no statistical significance; later on, at 45 and 60 min TF lowered significantly beneath control values. At a dose of 7.5 mg/kg Co chloride, the TF latency recorded a similar profile, while reaching statistical difference at 15, 30 and 45 min (fig. 1).

Calculations performed to evaluate pain inhibition showed that Co chloride is inducing a dose-dependent

antinociceptive effect while TF pain is reduced up to 30.87%. At 15 minutes, TF pain reduction by 30.87% was onset by a dose of 3.75 mg/kg while the 7.5 mg/kg dose induced the maximal inhibition of 24.31% at 15 min (table 1).

The intraperitoneal administration of Co chloride was evaluated by HP test and increased dose dependent latencies were observed at 12, 30, 45 and 60 min (fig. 1). Doses of 3.75 mg/kg and 7.5 mg/kg induced statistically significant effects at 15 and 30 min . Peak values were measured at 15 min for the 3.75 mg/kg dose (13.66±0.46 sec) and the 7.5 mg/kg dose (16.13±1.27 s).

Maximum possible effect deduced by HP latencies conversion into percent values showed an antinociceptive effect for Co chloride while pain is diminished by 22.46%. In HP test, pain was reduced by 8.03% for a dose of 3.75 mg/kg at 15 min while the maximal inhibition of 22.13% at 15 min was induced by the dose of 7.5 mg/kg.

Co chloride is inducing a statistically significant pain reduction illustrated by writing test results, on all recorded time intervals for all tested doses. Visceral pain undergone maximal inhibition at all times records for the 7.5 mg/kg doses (100%). Pain inhibition in our study was more important for the 7.5 (100% continuously) than for the 3.75 mg/kg dosage (67.86% at 30 min) (table 2).

Spontaneous behaviour changes were significant for the 3.75 mg/kg Co chloride dose, following evaluation by activity cage test. A mild sedative and anxiolytic effect was recorded.

Measurements performed for Ni chloride intraperitoneal administration (TF test) showed a significant latency at

Drug/time interval	5'	10'	15'	20'	25'	30'
Co chloride 3.75	42.06	45.45	36.57	47.69	40.09	67.86
Co chloride 7.5	100.00	100.00	100.00	100.00	100.00	100.00
Ni chloride 0.5	20.33	17.01	-0.12	7.58	2.95	7.14
Ni chloride 2	67.41	64.60	34.27	16.30	-3.04	-37.50
Sodium Molybdate 25	21.37	20.49	18.99	25.89	23.32	44.64
Sodium Molybdate 50	36.37	41.96	41.92	40.71	35.30	62.50

Table 2
AVERAGE PAIN INHIBITION VALUES FOR TESTED SUBSTANCES IN WT FOLLOWING ADMINISTRATION OF Co CHLORIDE 3.75 AND 7.5 mg/kg, Ni CHLORIDE 0.5 AND 2.0 mg/kg, SODIUM MOLYBDATE 25 AND 50 mg/kg.
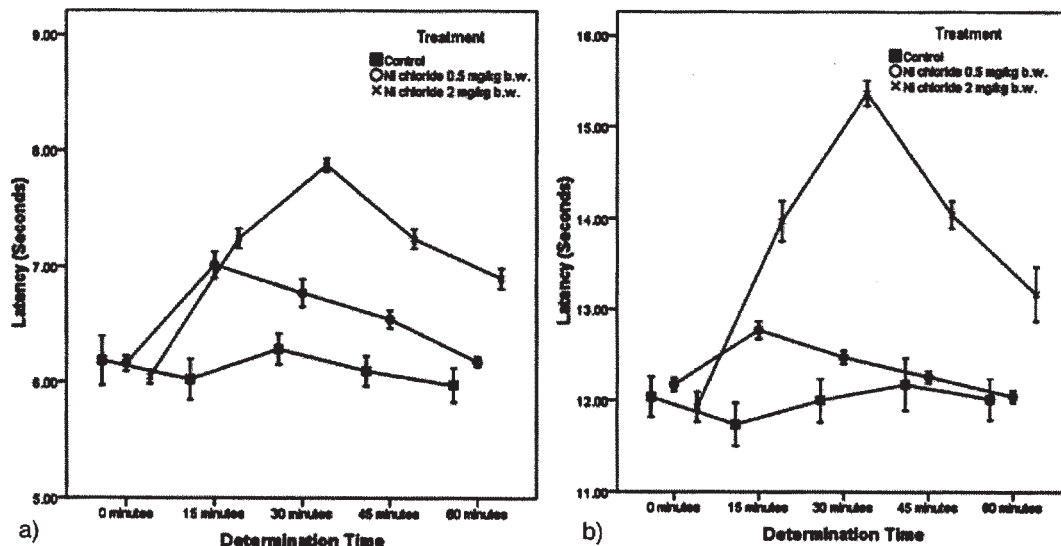

a)

b)

Fig. 2. a. Latencies for TF test, following Ni chloride intraperitoneal injection at different doses (0.5 and 2 mg/kg respectively).
b. Latencies for HP test, following Ni chloride intraperitoneal injection at different doses (0.5 and 2 mg/kg respectively)

15, 30, 45 and 60 min and indicated a significant antinociceptive effect. TF latencies were dose-dependent for the 0.5 mg/kg and 2 mg/kg doses at 15 and 30 min and the maximal effect recorded at 30 min led to a mean TF latency of 7.87±0.15 s (fig. 2). Conversion of TF latencies into a percent of maximum possible effect led to the observation that Ni chloride analgesic effect is dose-dependent and is expressed by TF pain decrease up to 20.41% (2 mg/kg dose, at 30 min) (table 1).

For the same salt, administered also intraperitoneally, HP test emphasized antinociceptive effects for all recorded time intervals and all doses while only some of the results are statistically significant. A dose-dependent effect was reflected by the response intensity. Even low doses (0.5 mg/kg) induced significant latencies at 15 min (that includes a peak of 12.77±0.26). Maximal intensity effect at 30 min was obtained by a higher dose of 2 mg/kg (peak at 15.37±0.36) (fig. 2). While converting HP latencies in percent of the maximal possible effect, Hi chloride was observed to induce a dose-dependent, antinociceptive effect, reducing the pain up to 19.01% (table I).

Spontaneous behaviour changes were significant for the 0.5 mg/kg Ni chloride dose, following evaluation by activity cage test at 0 - 15 min and for the 2 mg/kg dose at 0 - 25 min (table 2). No significant changes in the number of horizontal or vertical movements were recorded for a dose of 1 mg/kg Ni chloride.

Evaluation regarding sodium Molybdate effects following intraperitoneal injection by TF test showed an increase in TF latency at 15, 30 and 45 min, thus indicating a significant antinociceptive effect. TF latency increase expressing analgesic effect seems to be dose-dependent. A significant TF latency is observed at 15 min for a dose of 25 mg/kg sodium Molybdate. For a higher dose of 50 mg/kg, the latency profile is reproducible but with statistical significance at 15, 30 and 45 min (fig. 3).

A dose-dependent analgesic effect is induced by sodium Molybdate, and TF pain is lowered up to 13.40%, following pain inhibition calculations. TF pain was reduced by 8.79%

at 15 minutes for a dose of 25 mg/kg while the dose of 50 mg/kg is inducing a maximal inhibition of 12.42% at 15 min (table 1).

Sodium Molybdate intraperitoneal administration effect was evaluated also by HP test. Observed latencies were increased and dose-dependent at 15, 30, 45 and 60 min (fig. 3). Only at 15 and 30 min, recorded effects were statistically significant for all tested doses. Peak values were measured at 15 min for all doses (12.87 ± 0.14 s for the 25 mg/kg; 13.69 ± 0.32 s for the 50 mg/kg). Conversion of HP latencies into percent of maximum possible effect led to the observation that sodium Molybdate reduced the pain by 9.22% and induced an antinociceptive effect. HP pain was reduced by 4.07% at 15 minutes for a dose of 25 mg/kg while maximal inhibition of 9.22% was reached at 15 min for a dose of 50 mg/kg (table 1).

Spontaneous behaviour changes were significant for all doses and recorded time intervals and were expressed by the decrease of the total number of writhings. Maximum inhibition regarding visceral pain was observed at 30 min for all doses. Present results showed maximum pain inhibition as more significant for a dose of 50 mg/kg dose (62.50%) than for a dose of 25 mg/kg (44.64%) (table 2). A mild sedative effect is suggested by significant changes in horizontal axis movements for a dose of 50 mg/kg in the cage activity test (fig. 4).

Results and discussions
Cobalt

Our results both confirm and add to previous studies, showing for the first time the presence of a dose-dependent *in vivo* antinociceptive effect of Co chloride. Antinociceptive activity during thermoalgesic tests show an inhibition degree of 27-31% in TF and 18-40% in HP. Antinociceptive effect for Co chloride during WT is more present, ranging between 36 to 100%. The lower dose of Co chloride we used represents 1/24 of LD_{50}, thus limiting the potential toxic effects from interfering with our study.
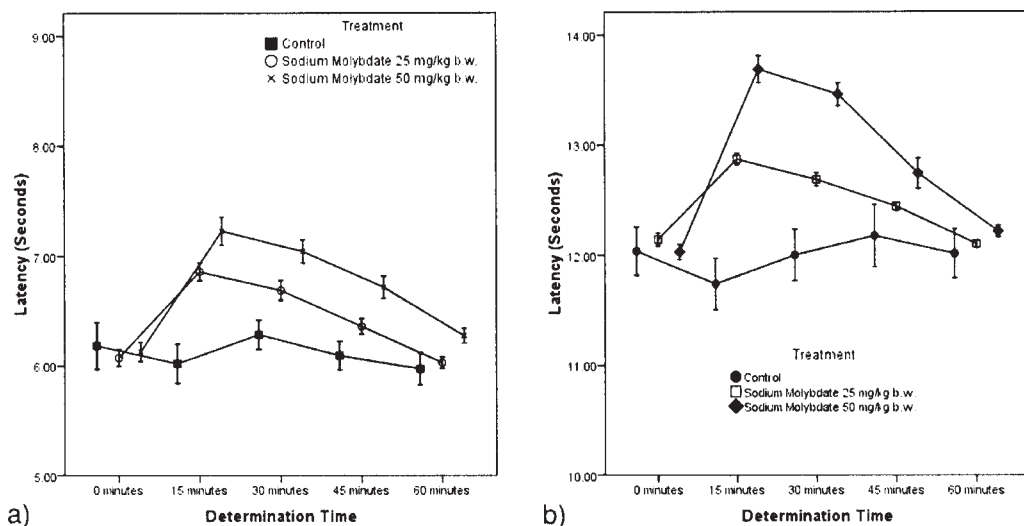
Fig. 3. a. Latencies for TF test, following sodium Molybdate intraperitoneal injection at different doses (25 and 50 mg/kg respectively). b. Latencies for HP test following sodium Molybdate intraperitoneal injection at different doses (25 and 50 mg/kg respectively)
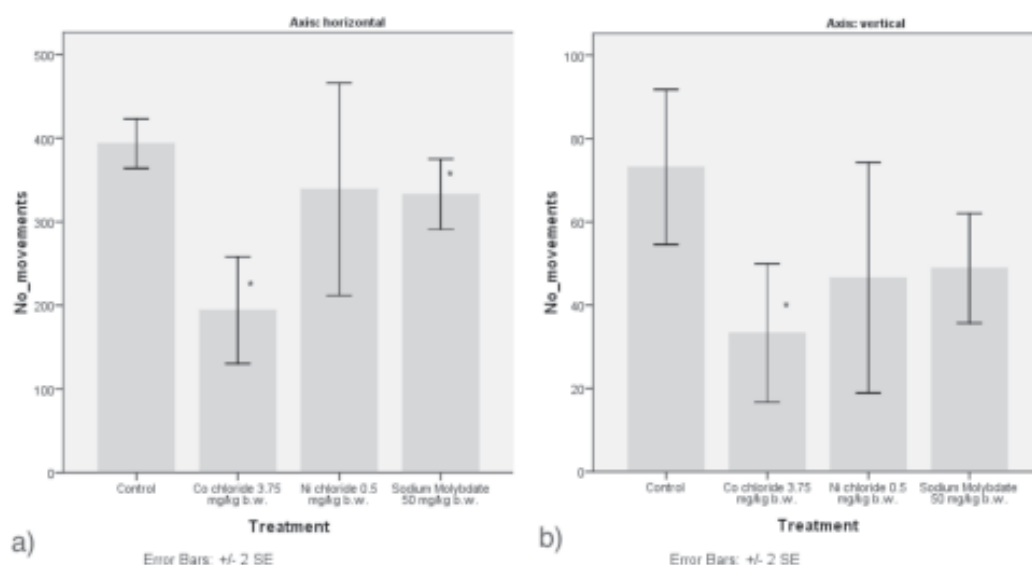

Fig. 4. a. Number of spontaneous movements per time interval in activity cage test, on horizontal ax, following administration of 0.9% saline for the control group, Co chloride 3.75 mg/kg, Ni chloride 0.5 mg/kg and sodium Molybdate 50 mg/kg. b. Number of spontaneous movements per time interval in activity cage test, on vertical ax, following administration of 0.9% saline for the control group, Co chloride 3.75 mg/kg, Ni chloride 0.5 mg/kg and sodium Molybdate 50 mg/kg

Spontaneous behaviour tests indicate both sedation and an anxiolytic effect in our experimental construct, inferring a possible toxic CNS effect [29-32]. However, the difference between the 100% nociceptive protection in visceral pain WT and the 31% effect in TF at the same time intervals and dose, dismisses as only possible explanation for our results the CNS depressant effect observed.

Co, Ni or Mn ions perfused in a Ca-deficient environment block synaptic transmission [28, 33] and microinjections of Co chloride in dorsal rat mesencephalon or the periapeductal gray, an area involved in visceral nociception, induce antinociceptive effects in rats [34, 35]. Co chloride also inhibits synaptic transmission at periapeducal grey level [36] and may act as a presynaptic blocker involved in nociception [37]. However, it appears that the mechanisms and involvement of this HTME in nociception is far more complex. Engelman et al. [38] used Co chloride to identify Ca permissive AMPA receptors in the dorsal horn of lamina I and lateral area of lamina II. In lamina I, Co-positive NK$_1$ receptors for P-substance are also present, concluding that they might play a role in nociception. Tong and MacDermott [39] observed that AMPA receptors contribute to the dorsal horn synaptic transmission and are involved in pain signaling pathways, making a Ca channel block through

Co replacement a possible explanation for the antinociceptive effect observed in our experiments. Similar to other metal trace elements (Zn, Ni, Cd, Mn), Co uses specific intracellular transporters [40], becoming cofactor for enzymes involved in the synthesis of neuropeptide and neuromediators but also in the superoxide metabolism. A functional connection between ACDP$_4$ transporter and the ionic chaperonin COX11 suggests specific intracellular sites for these metallic ions [41]. Valinoid and capsaicin-sensitive thermoreceptors were also thoroughly investigated using Co's ability to use calcium channels [42, 43] investigated the inductive role of cobalt-protoporphyrin on hem-oxygenase-1, known as a modulator for inflammatory pain, which might present novel pharmacological targets for analgesic drug development. The data available shows a complex role in pain for Co at various CNS levels, observations in agreement with our previous results [44, 45]. Furthermore, previous works lack in-vivo behaviour experimental data regarding Co and thus our results might represent a starting point for the presented subject.

Nickel

Acid-sensing ionic channels, present in central and peripheral neurons, with a specific role in nociception, neural hyperexcitability and hyperalgesia, can be inhibited in a dose-dependent manner by Ni ions [18, 46, 47]. Nelson et al. [48] insists on the Ni role on Ca channels according to their Zn affinity.

Our results show that Ni ion plays a moderate but significant and dose-dependent antinociceptive action in thermoalgesic tests, ranged from 9.7 to 20% between 15-30 min and effect persistence over 60 min for 2 mg/kg in HP test. WT show peculiar results – an initial short but significant antinociceptive action, followed by a hyperalgesic trend in the second part of the test. This biphasic aspect made us consider two different mechanisms; one mechanism involving a fast response while the second mechanism being delayed until the trace element arrives to some specific structures (channels, enzymes) whose impaired activity leads to the observed hyperalgesia. No significant changes during spontaneous behavior AC were observed. Poor literature data regarding Ni role in nociception allows us just few considerations regarding the mechanism and action place for this rare element in nociception. Prado [49] studying Ca^{2+} involvement in pain and antinociception, reported Ni and other cations (Ce^{3+}, La^{3+}, Cd^{2+}, Co^{3+}, Mg^{2+}, Mn^{2+}) as unselective blockers for the Ca^{2+} channel pore. It is also well-known that Ca^{2+} is required to induce nociceptive mediator delivery [12]. N and P/Q Ca^{2+} channels involved in nociception are found in dendrites and neuronal bodies. Some channel subtypes are also found in postsynaptic membranes of the glutamatergic neurons, involved in pain transmission. A more thorough study by Nelson et al. [48] describes Ni roles on Ca^{2+} channels and also their affinity for Zn^{2+} ions, showing that after a peripheral lesion, the nociceptive receptors become hyperexcitable. These receptors are also located in dorsal ganglia where neurons exhibit an increased number of T-type calcium channels. Ni ion was also proved to discriminate between various T-type Ca^{2+} channels, its affinity for Ca^{2+} 3.2 channel being 20 times higher compared to other isoforms. The antinociceptive effect of Ni could be similar to Zn, although this has a non-specific affinity for these channels. Intrathecal injections of Zn induce antinociceptive effects in mice, while Zn chelating agents lead to hyperalgesia [50]. Though current data may explain Ni antinociceptive action by Ca channels interference in peripheral nerve ends, the hyperalgesic effect observed in the second phase of the writing test to acetic acid remains to be further investigated.

Molybdenum

Mo showed significant antinociceptive effects in all our tests which are dose - independent and appear in a time range of 15 - 30 min from administration. The antinociceptive levels in the thermoalgesic tests were too limited to have clinical significance (inhibition values ranging between 8-13% for TF and 4-9% for HP). WT however, showed an antinociceptive activity of 33% at 15 minutes and 68% at 30 min, effect that is associated with a slight sedation (in AC). Current literature lacks data on in-vivo experimental data regarding Mo action on nociception. Toxicity studies in animals did not reveal any significant CNS effects nor motor dysfunctions. One pilot study [21] presented 14 patients undergoing pain symptoms that received $500 \mu g$ Mo in oral administration for 4 weeks and compared them to a placebo group. Significant pain relieve was recorded in Mo-treated group, together with general status improvement. Mo induced a growth latency of 50-78%, with visible cortico-dystrophies in a 6 weeks toxicity tests with oral doses of 0.2 mg, 7.5mg or 30 mg/kg/day in rats [4] with similar studies investigating Mo toxic effects on weight and hair color. We found no study regarding nociception or possible action mechanisms at cellular and subcellular level in pain. Mo is included in some Mo-hydroxylases family, enzymes involved in metabolism of some drugs in humans. These enzyme functions are not fully understood [50] but their effect on free radicals is generally accepted. Recent data seem to open a path toward more complex investigations, as chimeric animals (rats with human hepatic cells) were obtained to study various drug effects on Mo-enzymes in the cell [50] and also a potential interference of Mo with pro or anti-nociceptive molecules. Literature data show only a strong inhibiting effect of methadone on Mo-hydroxylases [51].

Spontaneous behavior test discussion

The spontaneous behaviour test was negative for nickel chloride, thus validating and completely eliminating any toxicity related contamination of the interpreted results. The test did show significant changes for Co and Mo. As explained above, while these changes may infer a possible toxic CNS effect or contamination of the anti-nociception studies, we maintain the conclusion (backed by the literature data too) that the difference between the 100% nociceptive protection in visceral pain WT and the 31% effect in TF at the same time intervals and dose, dismisses as only possible explanation for our results the CNS depressant effect observed. Had the anti-nociception effects been due *only* to the toxic or depressant CNS effect of the HMTE, the pain responses would have been similarly decreased in all pain tests performed. But this was not the case as clearly presented.

Limitations

Due to conflicting and limited in-vitro information about these HMTE's influence on pain, and the scarcity of previous in-vivo studies, the authors acknowledge the difficulty in choosing the test doses. The doses administered were calculated as fractions of known, published intraperitoneal IP LD50 in mice, and only balanced with the doses for various in-vitro studies as well as dietary supplements doses and recommendations. While considering potential toxic effects, the authors also had to look for doses in acute tests that were believed to generate a response on nociception, by extrapolating the literature in vitro doses rather than using the daily food intake. Thus, the doses used, and the fraction of LD50 they represent, are clearly within the limits for obtaining a therapeutic response in living organisms but without significant suspicion of toxicity. The way of administration was chosen IP because it provides the standard approach for an acute in-vivo test, with much better accuracy, dosing and control than oral administrations. Another critical point in our decision to use IP administration was the ethical committee of our institution that asked for previous existing data on these HMTE and pain.

Due to the scarcity of previous in-vivo studies and the difficulty in ethically justifying an oral long-term test with no previous literature data, we started our research from the beginning with the acute IP tests. Our experimental models used doses 10-20 times higher than dietary intakes, but still were a fraction of LD50's and did not induce significant toxicity, while clearly proving the effects that HMTE's produce on nociception.

Conclusions

The main purpose of this paper was to investigate the response to pain stimuli in several very straightforward tests, after the administration of HMTE. Our paper shows that Co has a moderate anti-nociceptive activity in thermoalgesic tests and superior values in chemoalgesic tests at higher doses, with some anxiolytic and sedative effects also recorded. Mo shows a variable antinociceptive activity in low doses, with a significant improvement for visceral pain. Behaviour tests showed that Mo can play a slightly sedative role. Ni chloride induces a moderate antinociceptive action and shows a biphasic effect in WT.

The conclusion of our paper is that all HMTE tested induced variable thermoalgesic and chemoalgesic antinociceptive effects after IP administration. These findings support further efforts to understand the molecular level effects of these trace metals. More important, our results represent a step forward in the investigation of how these daily HMTE may influence the traditional drug therapy in patients experiencing pain, when administered through daily food intake or dietary supplements.

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