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**BIOMEDICAL SCIENCES**

# Zinc deficiency disrupts pain signaling promoting nociceptive but not inflammatory pain in mice

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**Abstract:** Zinc (Zn) is an essential micronutrient involved in the physiology of nervous system and pain modulation. There is little evidence for the role of nutritional Zn alternations to the onset and progression of neuropathic (NP) and inflammatory pain. The study investigated the effects of a zinc restricted diet on the development of pain. Weaned mice were submitted to a regular (38 mg/kg of Zn) or Zn deficient (11 mg/kg of Zn) diets for four weeks, pain responses evaluated (mechanical, cold and heat allodynia; formalin- and carrageenan-induced inflammatory hypernociception), plasma and tissues collected for biochemical and metabolomic analysis. Zn deficient diet inhibited animal growth (37%) and changed mice sensitivity pattern, inducing an intense allodynia evoked by mechanical, cold and heat stimulus for four weeks. The inflammatory pain behavior of formalin test was drastically reduced or absent when challenged by an inflammatory stimulus. Zn restriction also reduce plasma TNF, increase neuronal activation, oxidative stress, indicating a disruption of the immune response. Liver metabolomic analyses suggest a downregulation of lipid metabolism of arachidonic acid. Zn restriction since weaned disrupts pain signaling considerably and reduce inflammatory pain. Zn could be considered a predisposing factor for the onset of chronic pain such as painful neuropathies.

**Key words:** zinc homeostasis, zinc deficiency, neuropathic pain, inflammatory pain, metabolomics.

## INTRODUCTION

Zinc is a trace element largely present on human body, being the second most abundant elements within human cells (Fraga 2005). It is an essential micronutrient involved in a large diversity of cellular metabolism, acting as a catalytic, co-catalytic or structural element of proteins and enzymes. DNA synthesis, cell division and immune response are some examples of biological processes that depends on zinc (Andreini et al. 2006).

The homeostasis of zinc is a vital process to the cell functions and physiology maintaining. Zinc is present in a wide variety of foods as nuts, meats and milk. Exogenous zinc is absorbed primarily in gastrointestinal system, followed by uptake in enterocytes and subsequent distribution through tissues. Endogenous levels of zinc are regulated by a refined system that involves two different families of zinc transporters proteins: ZnTs and ZIPs. The firsts are generally involved in sequestrate this ion into intracellular region or export zinc out of the cell when concentration is high, while ZIP proteins

are responsible to bring zinc into cell or release when zinc stores are low (Tepaamorndech et al. 2014).

The recommended daily ingestion of zinc to adults should be about 10 mg (Institute of Medicine 2005). However, about 17% of the world´s population consumes inadequate amounts of zinc (Wessells & Brown 2012). Delayed wound healing, male hypogonadism, mental lethargy, cell mediated immune dysfunctions and growth retardation are some of the several disorders associated with zinc deficiency (Prasad 1991, King 2011).

It has been demonstrated that zinc deficiency results in reduced circulating IGF-1 (insulin-like growth factor 1), which regulates cell cycle and division (Cossack 1991, Ninh et al. 1995). Furthermore, zinc acts also as a cofactor of tyrosine kinases, enzymes responsible to activate IGF-1 receptor (Wilson et al. 2012). A recent study of our group revealed that zinc restriction produces selective degeneration of the endocrine pancreas in mice (Sisnande et al. 2020). In the central nervous system (CNS) zinc is an essential micronutrient for cognition, development of memory and learning processes, as well as a neuromodulator of synaptic plasticity and an intracellular signaling transdutor (Hagmeyer et al. 2015, Jiang et al. 2022). Zinc is stored in presynaptic terminals of glutamatergic synapses, being co-stored with glutamate in specific vesicles and released from upon neuronal activation, mainly localized in cerebral cortex and in the limbic system. In these regions, high densities of a zinc-sensitive metabotropic receptor (ZnR) were detected, as well as ZnT and other proteins considered "zinc buffers" such as zinc import (ZIP) and metallothionein (MT) (Frederickson et al. 2005, Hara et al. 2017). Peripheral nervous system also counts with a variety of processes modulated by zinc. Zinc is present in thoracic and lumbar dorsal root ganglia (DRG), being also accumulated in sensory fibers (Velazquez et al. 1999).

It's well known that zinc plays important roles in pain modulation. Liu et al. (1999) showed that zinc ions delivered by intrathecal, local or systemic injection is able to reduce thermal hyperalgesia in rats with sciatic nerve injury. Nanomolar concentrations of zinc were able to block NMDA glutamate receptor, reducing neuronal activity in the spinal cord and providing an analgesic response in inflammatory and neuropathic pain models (Nozaki et al. 2011). A recent study also demonstrated that local delivery of zinc inhibits mechanical hypersensitivity evoked by paclitaxel-induced neuropathy, confirming the function of this micronutrient in different kinds of pain (Luo et al. 2017).

Beyond the direct modulation of exogenous zinc in nociceptive models, endogenous zinc levels also seem to be important to pain response. The reduction of zinc levels promoted by chelators agents presented a pro-nociceptive effect in different experimental models (Larson & Kitto 1997, Matsunami et al. 2011). In addition, some clinical studies also correlate painful conditions as fibromyalgia and myofascial pain with low levels of plasmatic zinc (Sendur et al. 2008, Barros-Neto et al. 2016). Acute painful episodes in children with sickle cell anemia was also associated with low serum zinc levels (Kudirat et al. 2019).

Despite of the growing knowledge about the role of zinc in the CNS and the influence of its dyshomeostasis on the development of degenerative diseases (Szewczyk 2013, Wojtunik-Kulesza et al. 2019), the scientific literature presents little evidence for the role of nutritional alternations of this metal to nociceptive disorders, such as neuropathic and inflammatory pain.

Considering the importance of zinc homeostasis for the nervous and immune system functioning, our hypothesis is that the imbalance of these systems is pivotal to the pathogenesis of pain. In the present study we aimed to investigate the impact of a zinc diet restriction in the onset and development of nociceptive disorders in mice since weaning.

**Abbreviations:** paw withdrawal threshold (PWT), dorsal root ganglions (DRGs), bovine serum albumin (BSA), Traveling Wave Ion Mobility Mass Spectrometer (TWIM-MS), glial fibrillary acidic protein (GFAP), activating transcription factor 3 (ATF-3), superoxide dismutase 1 (SOD1), Principal Component Analysis (PCA).

## MATERIALS AND METHODS

### Material

The diets were prepared by Rhoster® Ind. Com. LTDA (São Paulo). The Zn content in the diets were determined by mineral analysis (Laboratório de Ciência e Tecnologia, São Paulo, Brazil), revealing a zinc content of 38 mg/kg in the control diet and 11 mg/kg in the intervention diet (Supplementary Material). All other reagents were analytical grade and used according to the manufacturer.

### Experimental groups

Studies were performed using 21 days old male Swiss mice. Animals were housed at UFRJ's Faculty of Pharmacy vivarium, with free access to food and water in a 12-hour dark-light cycle. All experimental protocols were approved by the Ethical Animal Care Committee of Federal University of Rio de Janeiro (CEUA-UFRJ) under the number CCS-UFRJ-011/2015.

The animals (n=6-8 animals/group), immediately after weaning, were divided into two groups that received regular or zinc deficient diets and maintained with the assigned diets throughout the experiment. Control group was exclusively fed with the AIN-93 modified chow (with casein replaced by hen egg white solids, Salto's LTDA) (Reeves et al. 1993), as previously reported in detail (Sisnande et al. 2020) (Table SI). The diets were formulated with either regular mineral mix (AIN-93G-MX) or zinc-deficient mineral mix formulated without added zinc salts (AIN-93G-MX-ZnDef).

The body mass and mechanical allodynia were measured weekly. Tibial growth, thermal heat hyperalgesia and thermal cold allodynia were performed on the 4$^{th}$ week. Formalin and carrageenan evoked hyperalgesia tests were performed in independent groups.

### Behavior hypersensitivity tests

#### *Mechanical allodynia*

The mechanical threshold evaluation was performed as described by Decosterd & Woolf (2000). Briefly, animals were acclimated and then submitted to sequential mechanical stimulus ranging from 0.008 g to 2.0 g by *von Frey* filaments. The stimulus was applied under the left hind paw and each filament was presented 5 times to the paw, respecting a 60s gap between then. The mechanical threshold was defined as the last filament that obtained the highest number of paw withdrawal responses. Animals with a paw withdrawal threshold (PWT) higher than 2.0 g were excluded from the experimental groups (outliers). Mechanical sensitivity was measured weekly.

#### *Thermal heat hypernociception*

Animals were placed in a plantar thermal testing machine consisting in a contention chamber with a glass floor. After 30 min of acclimation, a beam of radiant heat is applied to the center of the hind paw, through a mobile heating source (high intensity light) positioned under the glass

floor. The heating stimulus was interrupted with the withdrawal of the paw (nociceptive response) and the time between the emission of light and the withdrawal of the paw was considered as the latency time (in seconds). To avoid plantar tissue damage, the upper cut-off limit was 20 sec. The left hind paw was evaluated three times, respecting an interval of five minutes, and the result expressed as the average of latency time readings (Hargreaves et al. 1988).

### *Thermal cold allodynia*

Cold allodynia was assessed by the acetone test, as previously described by Kawashiri et al. (2011). Briefly, mice were placed in an acrylic chamber with a metal grid floor that allows free access to the animal's paws. 30 min after the acclimation, 50 µl of acetone was sprayed onto the plantar skin and the nociceptive behavior (flinching and licking) time as recorded for five minutes.

### *Formalin-induced nociceptive behavior*

The formalin test was performed as previously described by Hunskaar & Hole (1987). After four weeks of diet intervention, control and zinc deficient groups received an intraplantar injection of formalin 2.5% (20 µL) on the right hind paw. The nociceptive behavior was recorded (licking time of the formalin-injected paw) for thirty minutes, first five minutes post-injection (neurogenic phase) and for fifteen minutes starting at the 15$^{th}$ min post-injection (inflammatory phase).

### *Carrageenan-induced hypernociception*

Inflammatory pain was evaluated using carrageenan-induced mechanical hypernociception assay. After four weeks of diet intervention, mice were injected with 50 µl of 1% carrageenan solution in sterile saline (NaCl 0.9%) into the sub plantar surface of the right hind paws. The mechanical sensitivity was evaluated using calibrated *von Frey* filaments. Mice were placed individually in acrylic cages with wire grid floors and the withdrawal response was determined at 0, 1, 3, 6 and 24 h post-challenge.

### Western Blot Analysis

Four weeks after diet intervention, the animals were euthanized, and the lumbar dorsal root ganglions (DRGs) were collected. DRGs samples were homogenized in lysis buffer (1% Triton, 20 mM Tris pH 7.6, 1 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 mM NaVO3) with protein inhibitor cocktail (SigmaFast, Sigma-Aldrich®) followed by total protein quantification. Protein samples were resolved in SDS-polyacrylamide and transferred to a nitrocellulose membrane. The membrane was then blocked with TBS buffer (25mM Tris-HCl pH 7,6, 0,2M NaCl and Tween 20 0,15%) + 3% BSA for 1 hour, to reduce unspecific ligation, followed by incubation with specific primary antibody - anti-SOD1 (Santa Cruz, RRID:AB_640874), anti-ATF-3 (Santa Cruz, RRID:AB_2258513), anti-GAP-43 (Santa Cruz, RRID:AB_640874) or anti-β-actin/actin (Santa Cruz, RRID:AB_2223228) - overnight at 4°C. After that, the anti-rabbit secondary antibody was incubated for 1 h and chemiluminescence measured using a ChemiDoc XRS + imaging system (Bio-Rad®) after development in chemiluminescence reagent (Westar Nova 2011, CYANAGEN). Bands intensity was determined using ImageJ software (NIH, United States).

### Plasma TNF quantification

To quantify the levels of TNF, blood was collected four weeks after the beginning of diet intervention by cardiac punction. Whole blood samples were centrifuged (10,000 rpm, 10 min at 4°C) and the plasma fraction collected to proceed the cytokine and protein measurements. TNF levels were determined using an enzyme-linked

immunosorbent assay kit commercially available (R&D Systems®).

## Metabolomics

### *Metabolite extraction*

Four weeks after diet intervention, the animals were euthanized, and the livers were collected. Liver samples were kept in liquid nitrogen until use. For metabolite extraction, specimens were transferred to 2 mL microtubes, and 1 mL of acetonitrile was added. Samples were mechanically disrupted with zirconia/silica beads (0.5 mm diameter) using a Mini-Beadbeater (1-min pulses, three times, with a 1-min rest on ice between pulses). The samples were centrifuged for 5 min at 16,000g and 4 °C and the supernatants were carefully transferred to new 2 mL tubes, dried in a speedvac evaporator and stored at -80°C for further analysis.

### *Mass spectrometry analysis*

Electrospray ionization–mass spectrometry (ESI–MS) analysis was performed in a Traveling Wave Ion Mobility Mass Spectrometer (TWIM-MS, Synapt G1 HDMS, Waters, UK). Dried samples were solubilized (10 µL of 60% acetonitrile for each mg of initial liver sample), kept in an ultrasonic bath for 5 minutes, and centrifuged at 14,000 rpm and 6 °C for 30 minutes. Each sample was divided into two parts for analysis conditions in positive and negative mode. For analysis in positive mode was added formic acid at 0.1% and for negative mode ammonium hydroxide at 0.5%. The quality control analysis was performed with a mixture of all samples which were injected at the beginning and the end of the analysis. Mass calibration was performed with phosphoric acid solution in range from 50 to 2,000 m/z. Samples were injected at a rate of 10 µL $min^{-1}$, data were acquired over the range from 100 to 1,000 m/z during 5 minutes in dynamic mode and scan time of 0.8 seconds. The positive ESI analysis was performed with a capillary voltage of 3.0 kV, sampling and extraction cone respectively at 50 V and 4.0 V. The settings for negative analysis were capillary voltage of 2.5 kV, sampling and extraction cone respectively at 50 V and 5.0 V. The nanoflow gas was set at 500 L h-1 at a desolvation temperature of 150 °C, and the source temperature set at 100 °C. All the acquisition of data was operated by Waters MassLynx v4.1 software.

Mass spectrometric data were processed using integrated MassLynx™ tools and MarkerLynx™ XS Application Manager from Water™s (Milford, MA, USA). Firstly, raw data were centroided using the *Accurate Mass Measure* tool and then the resulting mass data was processed using MarkerLynx™. The method parameters were as follows: analysis type = combined scan range, peak separation (Da) = 0.02 and marker intensity threshold (counts) = 2,000. Data were normalized to the total marker intensity and a two-dimensional data matrix (m/z versus peak intensity) was generated for each ionization mode and exported to a format amenable for further data analysis. Data from positive and negative modes were combined and *Principal Component Analysis* (PCA) was performed using the freely available software *Multibase* (http://www.numericaldynamics.com). To identify differences in metabolite composition between groups and to assign possible metabolite identities to m/z values showing at least a 2-fold difference in intensity between sets of samples, we manually calculated the mean and standard error of mean (SEM) for each metabolite, and data were queried against PATHOS – *The metabolomics tool from Glasgow Polyomics* (http://motif.gla.ac.uk/Pathos/index.html) (Table SII and Figure S2).

### Data Analysis

Statistical analyses were performed using GraphPad Prism ver 7.01 (GraphPad Software). Two-way ANOVA, One-way ANOVA or *t*-test was used depending on the experiment. Bonferroni and Tukey post-tests were used for multiple comparison analysis. A *p*-value of <0.05 was considered significant.

## RESULTS

### Reduction of zinc intake since the weaning affects mice growth and body weight gain

Animals fed with control diet showed typical pattern of growth as indicated by gain in body weight (p<0.001) (Figure 1a). In contrast, zinc deficiency results in significant growth restriction of the animals evidenced by both, the reduced body weight (control = 23.2 ±1.4 g *versus* Zn-def = 14.9 ±1.1 g, 28 days after diet intervention; p<0.001) (Figure 1a), which does not present a significant difference with the respective baseline value, and tibia length (p=0.0004) (Figure 1b). Zinc deficiency also resulted in bowel malfunction, repetitive jumping behavior, hair loss and an increase of mortality (*data not shown*), confirming the impact of zinc deficiency to animal health development.

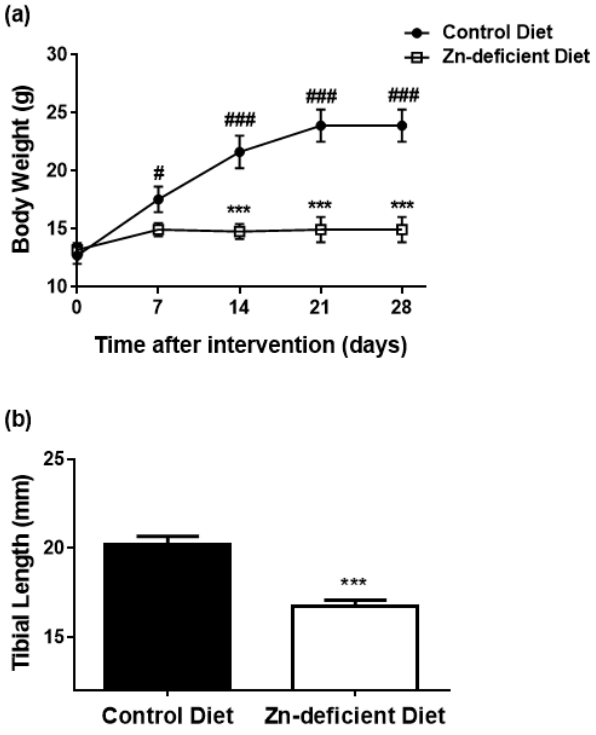


**Figure 1. Effect of dietary zinc deficiency on mice body weight and growth. (a) Control (filled circle) and zinc-deficient (opened square) diet animals body weights were measured weekly. (b) Tibial length sizes were measured at week 4. Data are expressed as mean ± SEM, n=9-15 animals, One-way ANOVA followed by Bonferroni post-test (body weight) and *t*-test (tibia length), ***p<0.001 vs control diet group, #p<0.05 and ###p<0.001 vs its baseline value.**

### Zinc deficiency increases nociceptive behavior evoked by mechanical and thermal stimulus

We further evaluated the basal nociceptive sensitivity against both mechanical and thermal stimulus (Figure 2). Basal mechanical sensitivity was measured weekly by the *von Frey* test (Chaplan et al. 1994). The response to thermal heat and cold stimulus was evaluated four weeks after the start of diet intervention by Hargreaves and acetone tests, respectively.

Young mice submitted to a control diet presented a low mechanical threshold that increased over the weeks along with growth (Figure 2a), which is a normal pattern observed in animal development, reaching a threshold of response compatible with a healthy young adult animal at the end of the experiment (McKelvey et al. 2015). In opposite, animals under zinc restriction showed a dissimilar pattern with the mechanical threshold consistently remained low throughout the development and growth of animals. After three weeks under dietary intervention, the control group showed a mechanical paw withdrawal threshold (PWT) of about 0.632±0.089g while the zinc-restricted group showed a PWT of about 0.379±0.061g (p=0.026). This intense mechanical allodynia remains until the 4th week (control 0.786±0.08g versus Zn-def 0.237±0.04g; p<0.001).

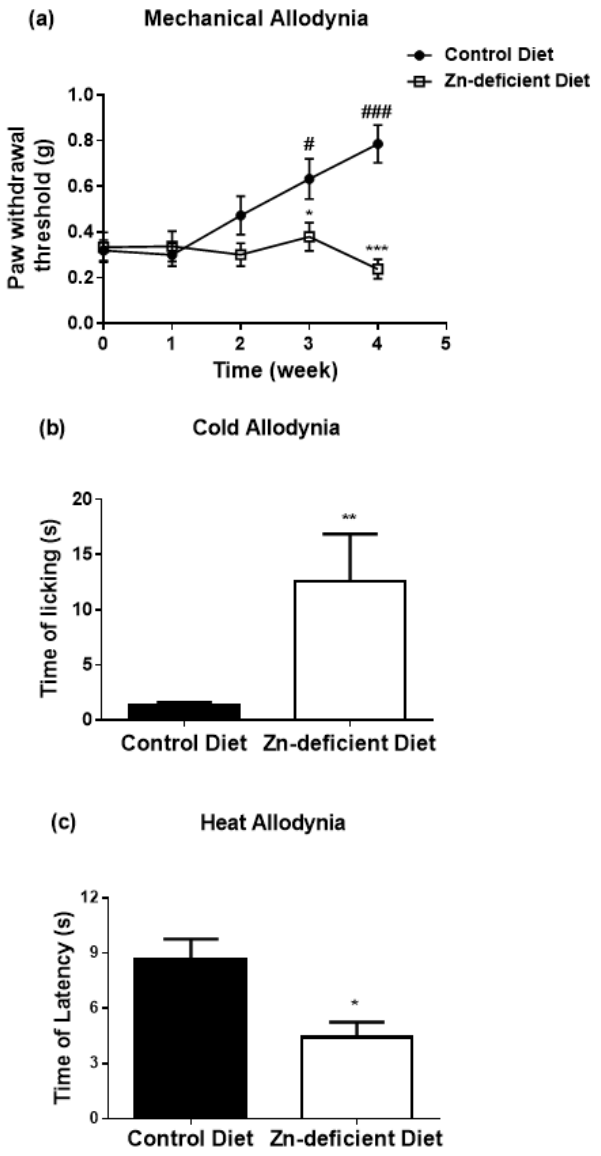


**Figure 2. Effect of dietary zinc deficiency on mice mechanical and thermal allodynia. (a) Control (filled circle) and zinc-deficient (opened square) diet groups mechanical withdrawal threshold were measured weekly by *Von Frey* filaments. (b, c) Cold and heat sensitivity, respectively, were measured at week 4. Data are expressed as mean ± SEM of animal's paw withdrawal threshold (g) (n=10-14), time of licking (s) (n=10-14) or latency time (s) (n=4-5), Two-way ANOVA followed by Bonferroni post-test (mechanical allodynia) and *t*-test (thermal allodynia), *p<0.05, **p<0.01 and ***p<0.001 vs control diet group, #p<0.05 and ###p<0.001 vs its baseline value.**

Mice submitted to a zinc deficient diet also presented a significant hypersensitivity to thermal stimulus after four weeks. The zinc-restricted group showed an increase in the nociceptive behavior evoked by cold stimulus (p=0.0048) (Figure 2b) and a reduction of the time to respond to heat innocuous temperatures compared to control group (p=0.022) (Figure 2c).

## Zinc deficiency increases neurogenic pain but abolishes inflammatory pain behavior

Since the dietary zinc restriction resulted in disrupted basal sensitivity, we hypothesized whether the hyperalgesic response could also be altered. To address this question, we performed the formalin inflammatory test (Hunskaar & Hole 1987).

The intraplantar administration of formalin induces two phases of pain behavior: neurogenic phase (0 to 5 minutes) and inflammatory phase (15 to 30 minutes) after injection.

Mice under zinc-deficient diet for four weeks showed an increase of the nociceptive behavior (licking time) in the neurogenic phase. The hypernociceptive response was about 50% higher in zinc-restricted mice than in regular diet group (p=0.0038). However, in the inflammatory phase, a significant reduction in the nociceptive response was observed for restricted zinc diet compared to control diet (p=0.0128) (Figure 3a).

Considering that zinc restriction affected inflammatory pain in formalin test, we hypothesized if the reduction of zinc intake could also affect pain behavior after intraplantar injection of carrageenan.

Animals consuming control diet presented a significant reduction of mechanical PWT one hour after the inflammatory stimulus. The hyperalgesic behavior remains even twenty-four hours after carrageenan injection (p<0.001) (Figure 3b). Zinc deficiency results in a low basal mechanical sensitivity (Figure 2a). The

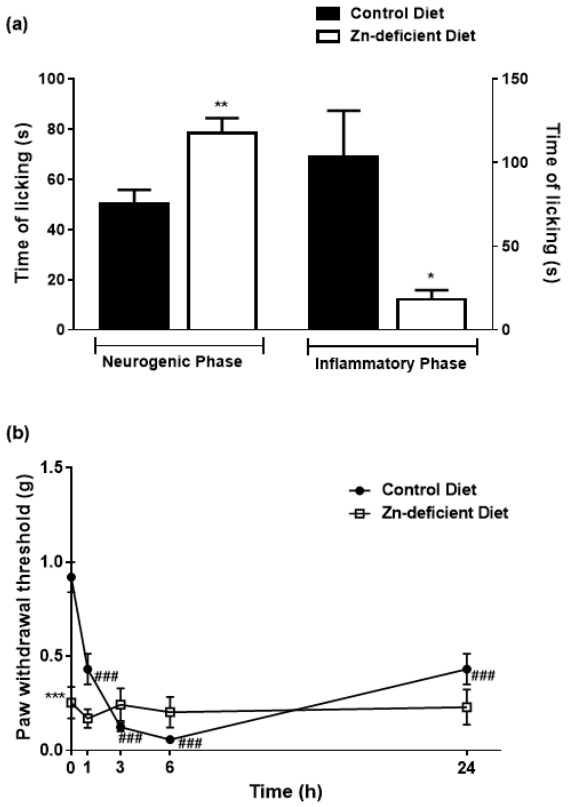


**Figure 3. Effect of dietary zinc deficiency on formalin induced nociception and mechanical hyperalgesia induced by carrageenan in mice. (a) Neurogenic and inflammatory phases of the formalin test (n=7-9). (b) Carrageenan induced hyperalgesia test (n=5-6). Time of licking (s) and paw mechanical withdrawal threshold (g) of control (filled bar/circle) and zinc-deficient (opened bar/square) diet groups were accessed at week 4. Data are expressed as mean ± SEM, *t*-test (licking time vs control diet group) and Two-way ANOVA followed by Bonferroni post-test (paw withdrawal), *p<0.05, **p<0.01 and ***p<0.001 vs control diet group, ###p<0.001 vs its baseline value.**

intraplantar administration of carrageenan did not alter the basal value of mechanical sensitivity (Figure 3b). PWT remained low, like its baseline and unchanged for twenty-four hours.

## Reduction of zinc intake changes the expression of proteins related to glial satellite cell, neuronal activation and oxidative stress in DRG

To understand the mechanisms behind the hypernociceptive behavior shown by animals submitted to zinc restriction in dietary, we attempted to estimate the dorsal root ganglion (DRG) levels of proteins related to glial satellite cells (GFAP, *glial fibrillary acidic protein)*, neuronal activation (ATF-3, *activating transcription factor 3*) and oxidative stress (SOD1, *superoxide dismutase 1*). A wide variety of neuronal stress conditions leads to activation of glial satellite cells and neurons, which can be inferred by the evaluation of GFAP and ATF-3 expression, respectively (Galbavy et al. 2015, Malaspina et al. 2010).

DRGs were removed four weeks after diet intervention and submitted to western blot analysis. DRGs of animals under dietary zinc restriction presented a significant reduction of GFAP levels (p=0.035) (Figure S1a) accompanied by increased ATF-3 level (p=0.0003) (Figure S1b), suggesting that neuron activation and damage may occurs independently of satellite cells activation. Aiming to understand if oxidative stress could be related with neuron activation and hypernociceptive disorders observed upon dietary zinc restriction, we also evaluate the levels of SOD1, an antioxidant enzyme that plays important role on oxidative stress and neuronal damage control. We have found the SOD1 levels apparently increased in the DRG of zinc-deficient mice compared to control group (p=0.049) (Figure S1c).

## Zinc-deficient diet decreases TNF plasma levels

The impact of zinc deficiency on inflammatory pain and hyperalgesia (Figure 3) led us to investigate whether the downregulation of the inflammatory process was occurring systemically, evaluating the levels of the pro-inflammatory cytokine TNF.

Animals under zinc-deficient diet showed reduced plasma levels of TNF compared to mice fed control diet (p=0.0016) (Figure 4).

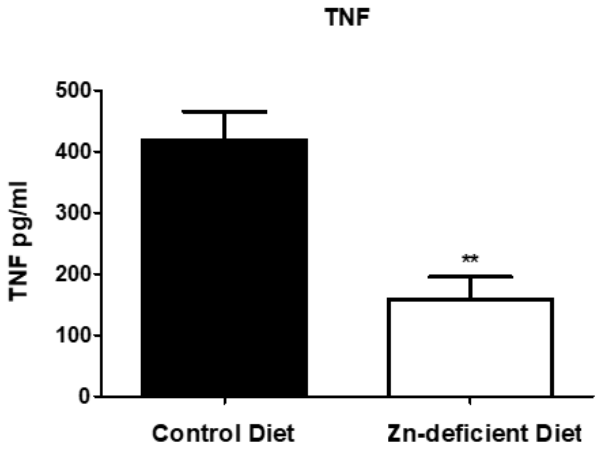


**Figure 4. Zinc-deficient diet effect on TNF plasma levels. TNF plasma levels were measured four weeks after starting the diet. Data are expressed as mean ± SEM of TNF in pg/ml, n=3 animals, *t*-test, **p<0.01 vs control diet group.**

### Zinc deficiency downregulates arachidonic acid (AA) metabolism in liver

To understand changes in metabolic profile due to zinc deficiency, we further performed liver metabolomics by mass spectrometry. A total of 3,021 different *m/z* (metabolic features) were found from both control and experimental groups, detected from combined positive and negative ion modes by ESI-QTOF-MS (Table I). A *Principal Component Analysis* (PCA) loading and score plots illustrates the extensive differences in liver metabolic composition between control and zinc deficient mice (Figure *S2*).

To investigate which of the metabolites detected were present at different levels in those groups of samples, the average intensities of all metabolites were calculated and results from each of the sample groups (control and Zinc-deficient diet) were compared. Metabolites that showed changes of 2-fold or more were used for further analyses. Based on this analysis we found that 767 out of the total 3,021 metabolites were present at different levels and changed by the diet when comparing samples from different diets (Table I), representing 25.4% of all detected

**Table I. Liver metabolites quantification of control and zinc deficiency diet mice.**

| Overview of ESI-QTOF-MS results | |
|---|---|
| ***Metabolites detected*** | ***Number of metabolites*** |
| Negative ionization | 1,177 |
| Positive ionization | 1,844 |
| Total | 3,021 |
| ***Metabolites changed*** | |
| Control > intervention | 560 |
| Intervention > Control | 207 |
| Total changed | 767 |
| % total | 25.4% |

m/z, suggesting an extensive metabolic shift due to zinc deficiency.

The metabolic pathways most significantly disturbed in livers and that differ between the two groups were illustrated in Figure 5. Although many metabolic pathways were affected, our data suggest that the arachidonic acid (AA) metabolism is markedly modulated due to zinc-deficient diet, presenting lower levels of many of its metabolites when compared to the control. Two putative metabolites of AA pathway showed increased levels in that experimental group (TXB2 and 6-Keto PGF1α) and only one showed no difference between the two groups (LTD4), as depicted in the AA metabolic pathway (Figure 6). Although the AA itself was not detected experimentally, several m/z corresponding to an array of potential AA derivatives were detected in lower levels (two-fold or higher) in mice livers with zinc restriction.

## DISCUSSION

In the present study, we showed that zinc deficiency due to dietary zinc restriction since weaning results in nociceptive disorders that are independent of some classical pro-inflammatory and pro-algesic mediators.

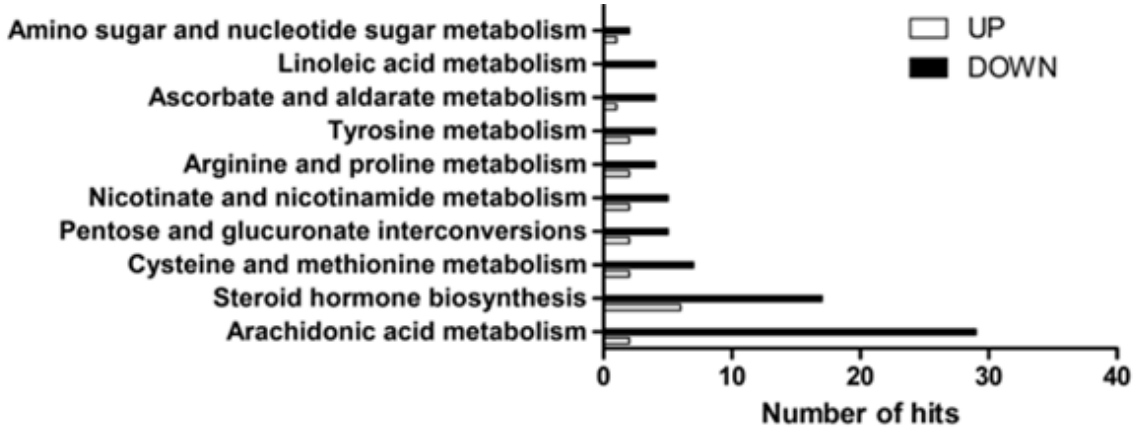


**Figure 5. Metabolomics analysis of liver from control and zinc-deficient mice. Total number of hits from metabolomic pathways altered upon intervention with zinc-deficient diet. Ions detected from electrospray analysis in both positive and negative modes were searched in the KEGG database and represented here for those that increased (opened bar) or decreased (filled bar) compared to animals under control diet.**

Zinc is recognized as a fundamental ion for proteins and enzymes. Several studies have shown zinc as an essential micronutrient for the development of memory and learning processes (Frederickson et al. 2005, Hara et al. 2017). In recent years, several studies on the role of zinc in the development and functioning of the nervous system have pointed out and highlighted $Zn^{2+}$ as a modulator of neuronal signaling (Sensi et al. 2011).

Zinc levels play important role in painful and neurodegenerative conditions, as observed in spinal cord injuries models, modulating the spinal cord signaling of pain (Ma & Zhao 2001, Su et al. 2012). Despite to the growing evidence of the zinc participation in pain modulation, the knowledge about the consequences of zinc deficiency and dyshomeostasis to pain disorders and its mechanisms are still unclear. Here in we showed for the first time the impact of diet zinc deficiency to mice development nociceptive disorders with an unanticipated decrease of inflammatory response pattern and metabolism.

Diet low in zinc showed to have great impact on the development and growth of the animals as expected and according to previous knowledge (King 2011, Jing et al. 2015). Our study showed that dietary zinc restriction increases nociceptive behavior evoked by mechanical and thermal stimulus, also increasing allodynic behavior evoked by cold temperatures, and the licking time of the neurogenic phase of the formalin test, whereas it almost abolishes the pain behavior of the inflammatory phase. These data corroborate previous findings on zinc modulation of pain evoked by different stimulus (Liu et al. 1999, Nozaki et al. 2011, Larson & Kitto 1997). The painful behavior of neurogenic phase is the result of nociceptors activation by the chemical agent followed by a massive contribution of central sensitization (Lebrun et al. 2000). Zinc is an endogenous regulator of excitatory neurotransmission, described as an allosteric modulator of NMDA receptors at spinal cord and able to attenuate C fiber-evoked potentials (Nozaki et al. 2011, Ma & Zhao 2001). These evidences suggest that pro-nociceptive effects observed at neurogenic phase of formalin test, as well as the other painful behaviors observed in this work for zinc-deficient mice, are due to the loss of physiological control of pain by endogenous zinc.

The inflammatory phase of the formalin test is characterized by the release and mobilization

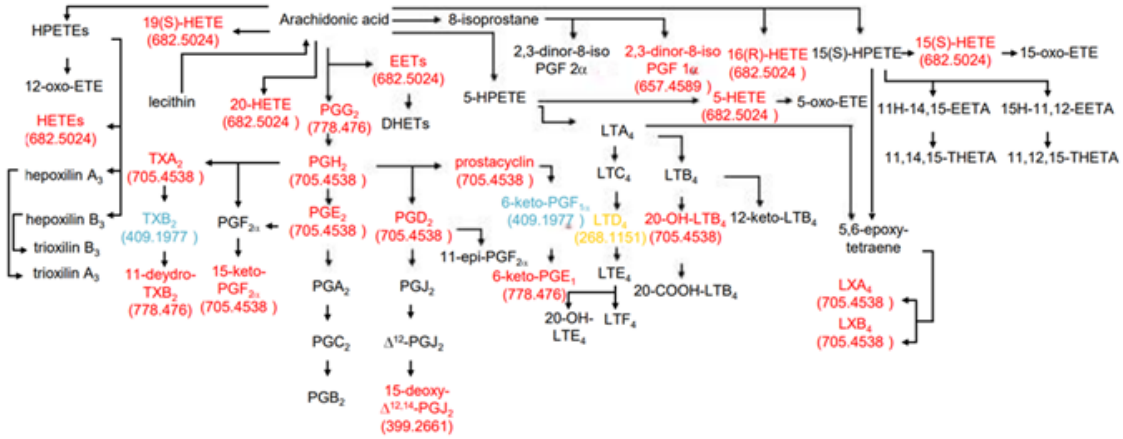


**Figure 6. Effect of dietary zinc deficiency on the arachidonic acid metabolism in mice. Schematic overview of the arachidonic acid metabolism pathway is show, depicting the metabolites increased (blue), decreased (red) or unaltered (black) in relative abundance upon dietary zinc restriction compared to animals fed with control diet. Numbers in parentheses represents the true *m/z* detected (for the M:H) ion.**

of a wide array of signaling molecules and inflammatory mediators that support the nociceptive response (Basbaum et al. 2009). Zinc is well recognized as an important factor to immune response regulation. Zinc deficiency has been associated with decrease of macrophages and neutrophils chemotaxis, phagocytosis and oxidative stress. Moreover, zinc levels seem to be important for the release of pro-inflammatory cytokines by macrophages (Haase et al. 2008, Ryu et al. 2011). In this sense, our findings suggest that the reduction in the hypernociceptive behavior observed for the zinc deficient group in the formalin inflammatory phase occurs because the immune system response of these animals is compromised. Zinc deficiency also keeps the paw withdrawal threshold reduced and unchanged facing an inflammatory insult such as in mechanical hypersensitivity evoked by carrageenan, reinforcing the absence of an inflammatory response in this condition. Furthermore, the inflammatory marker TNF was reduced, providing further evidence that nociceptive symptoms due to zinc deficiency in mice are not related to the increase of pro-inflammatory cytokines.

The reduced serum level of TNF suggests that nociceptive symptoms observed are not dependent of peripheral TNF signaling. Additionally, we also observed in DRG of mice submitted to zinc restriction, a reduction of glial activation, contrary to what is described in classic chronic pain conditions (Galbavy et al. 2015), as well as an increase of neuronal activation and oxidative stress, despite of glial cells downregulation. Taken together, these findings reinforce the idea of an immune response and oxidative stress disruption of the sensory neurons during zinc deficiency conditions.

Metabolomic analysis of mice livers indicated that zinc deficiency from birth resulted in reduction of several metabolites of the arachidonic acid (AA) pathway that corroborate and explain the lack or reduced inflammatory pain behavior. Low zinc diet has been reported to contribute to an inflammatory state and to a reduced liver arachidonic acid level (Bettger et al. 1979, Dieck et al. 2005). The impact of liver arachidonic acid reduction could be expanded to plasma and tissues surrounding nociceptors, affecting production of prostanoids which sensitizes these cells. The AA metabolism generates a diverse class of bioactive lipid

mediators involved in inflammatory and pain processes. Indeed, the relatively high number of metabolites of AA pathway that were identified by our exploratory method, suggests that this pathway is an important component for the immune response disruption promoted by dietary zinc restriction.

Our findings reinforce the impact of dietary zinc in maintaining homeostasis of sensory transmission, highlighting zinc deficiency as a predisposing factor for the onset of chronic pain such as painful neuropathies.

In conclusion, this study provided new evidence on the role of zinc on pain development, other than the analgesic effect reported previously, emphasizing its nutritional importance and the relevance as a dietary supplementation, which requires further clinical human studies.

### Data Availability Statement (DAS)

All datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## SUPPLEMENTARY MATERIALS

**Figures SI-SII.**
**Tables SI-SII.**

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