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**Chronic HgCl<sub>2</sub> treatment increases vasoconstriction induced by electrical field stimulation. Role of adrenergic and nitrergic innervation.**

Running title: Role of vascular innervation in HgCl<sub>2</sub>-treated rats.

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## Abstract

**Objectives:** We investigated the possible changes in rat mesenteric artery vascular innervation function caused by chronic exposure to low doses of mercuric chloride ( $\text{HgCl}_2$ ), as well as the mechanisms involved.

**Methods:** Rats were divided into two groups: control and  $\text{HgCl}_2$ -treated rats (30 days, 1st dose 4.6  $\mu\text{g}/\text{kg}$ , subsequent dose 0.07  $\mu\text{g}/\text{kg}\cdot\text{day}$  im). Vasomotor response to electrical field stimulation (EFS), noradrenaline (NA) and nitric oxide (NO) donor DEA-NO were studied, neuronal NO synthase (nNOS) and phosphorylated nNOS (P-nNOS) protein expression were analysed and NO, superoxide anions ( $\text{O}_2^-$ ) and NA releases were also determined.

**Results:** EFS-induced contraction was higher in the  $\text{HgCl}_2$ -treated group. 1  $\mu\text{mol}/\text{L}$  phentolamine decreased the response to EFS to a greater extent in  $\text{HgCl}_2$ -treated rats.  $\text{HgCl}_2$  treatment increased vasoconstrictor response to exogenous NA and NA release. 0.1 mmol/L L-NAME increased the response to EFS in both experimental groups but the increase was greater in segments from control animals.  $\text{HgCl}_2$  treatment decreased NO release and increased  $\text{O}_2^-$  production. Vasodilator response to DEA-NO was lower in  $\text{HgCl}_2$  animals. Tempol increased DEA-NO-induced relaxation to a greater extent in  $\text{HgCl}_2$ -treated animals. nNOS expression was similar in arteries from both experimental groups, while P-nNOS was decreased in segments from  $\text{HgCl}_2$ -treated animals.

**Conclusion:**  $\text{HgCl}_2$  treatment increased vasoconstrictor response to EFS as a result, at the least, of reduced NO bioavailability and increased adrenergic function. These findings offer further evidence that mercury, even at low concentrations, is an environmental risk factor for cardiovascular disease.

*Key words:*  $\text{HgCl}_2$ , vascular innervation, nitric oxide, noradrenaline.

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## Introduction

Adverse effects of mercury exposure usually result from acute or chronic exposure to elemental mercury vapor, inorganic mercury salts or organic mercury compounds such as methylmercury. The toxic effects of mercury compounds have been widely described to cause damage to the nervous system and kidneys [1-4]. Several studies reported a dose-dependent toxic effect from mercury leading to death at high concentrations [1-4]. On a biochemical level, mercury is known to interact with SH groups of proteins, altering their activities [5]. Effects that depress ATP hydrolysis and Na<sup>+</sup> K<sup>+</sup>-ATPase activity have been described in nervous tissue and brain microsomes [6-10]. Some epidemiological studies have indeed found an association between increased levels of mercury in the body and a risk of cardiovascular disease [11-13]. In fact, acute mercury exposure reduces the development of myocardial force [14] and inhibits myosin ATPase activity [15]. Moreover, chronic mercury exposure increases vascular resistance and induces hypertension [12, 16].

In animal and human studies mercury increases free radical production, oxidative stress [17-19] and lipid peroxidation [20], promotes dyslipidemia [11], affects antioxidant defences such as glutathione, superoxide dismutase and catalase [21, 22], and alters immune [23] and endothelial function [18, 24]. We have previously demonstrated that both acute and chronic administration of low doses of mercuric chloride (HgCl<sub>2</sub>) increase phenylephrine-induced contractions and decrease endothelium dependent vasodilator responses in different vascular beds [19, 25]. Endothelial dysfunction is known to occur in mercury-treated animals as a result of the decreased NO bioavailability induced by increases in O<sub>2</sub><sup>-</sup> production from NADPH oxidase [25].

Vascular tone is also determined by an equilibrium among several neurotransmitters released from nerve endings [26, 27]. We have previously reported that rat mesenteric arteries possess adrenergic, nitrenergic and sensory innervation whose role may change depending on the physiopathological situation [28-30]. Electrical field stimulation (EFS) produces a vasoconstrictor response, which is the integrated result of neurotransmitter release, mainly of noradrenaline (NA), nitric oxide (NO) and calcitonin gene-related peptide (CGRP) [31, 32]. Some reports have shown that mercury also affects secretion of neurotransmitters from neural tissue [33, 34]; however, there are no reports describing the effects of mercury on vascular innervation.

The aforementioned results have led us to hypothesise that chronic mercury exposure could alter the functional role of vascular innervation. Therefore, the goal of this work was to study the effect of chronic exposure to low doses of HgCl<sub>2</sub> on adrenergic, nitrenergic and sensory innervation in mesenteric artery as well as the mechanisms involved in these effects.

## Materials and Methods

### *Animals*

Male Wistar rats (3 months-old) were obtained from the Animal Quarters of the Universidad Autónoma de Madrid (Registration number EX-021U). All animals were housed in the Animal Facility of the Universidad Autónoma de Madrid (Registration number EX-021U) in accordance with guidelines 609/86 of the E.E.C., R.D. 233/88 of the Ministerio de Agricultura, Pesca y Alimentación of Spain, and Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health [NIH publication No. 85.23 revised 1985]. All experimental procedures involving animal use were approved by the Ethics Committee of the Universidad Autónoma de Madrid.  $\text{HgCl}_2$  solution was prepared and used, and animal tissue manipulation was performed following the appropriated security measures. Rats were housed during treatment at a constant room temperature, humidity, and light cycle (12:12 h light-dark) with free access to tap water and fed with standard rat chow ad libitum. Rats were divided into two groups: control (0.9 % saline solution im) and  $\text{HgCl}_2$  treated for 30 days (im injection of  $\text{HgCl}_2$  dissolved in a saline solution vehicle inside a gas chamber, 1st dose 4.6  $\mu\text{g}/\text{kg}$ , subsequent dose 0.07  $\mu\text{g}/\text{kg}\cdot\text{day}$  to cover daily loss). Systolic blood pressure was indirectly measured in awake animals by the tail-cuff method, as previously reported [35] (Letica, Digital Pressure Meter, LE5000, Barcelona, Spain).

Animals were sacrificed by  $\text{CO}_2$  inhalation 30 days after initiating treatment; the first branch of the mesenteric artery was carefully dissected, cleaned of connective tissue and placed in Krebs-Henseleit solution (KHS, in mmol/L: NaCl 115,  $\text{CaCl}_2$  2.5, KCl 4.6,  $\text{KH}_2\text{PO}_4$  1.2,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1.2,  $\text{NaHCO}_3$  25, glucose 11.1,  $\text{Na}_2\text{EDTA}$  0.03) at 4° C for protein expression analysis; some arteries were rapidly frozen in liquid nitrogen and kept at -80°C until the day of analysis.

### *Mercury contents*

The mercury levels in acid-digested samples of whole blood were measured at 30 days of treatment in duplicate by atomic fluorescence spectrometry (PSA Analytical, Model 1025 Millenium System) in the Centro de Espectrometría Atómica (Universidad Complutense de Madrid). The accuracy of the mercury analysis was checked with an external reference material (theoretical value, 3.8 ng/ml; experimental value, 3.85 ng/ml).

### *Vascular Reactivity*

The method used for isometric tension recording has been described in full elsewhere [31, 36]. Two parallel stainless steel pins were introduced through the lumen of the vascular segment: one was fixed to the bath wall, and the other connected to a force transducer (Grass FTO3C; Quincy, Mass., USA); this, in turn, was connected to a model 7D Grass polygraph. For EFS experiments, segments were mounted between two platinum electrodes 0.5 cm apart and connected to a stimulator (Grass, model S44) modified to supply adequate current strength. Segments were suspended in an organ bath containing 5 mL of KHS at 37° C and continuously bubbled with a 95%  $\text{O}_2$  to 5%  $\text{CO}_2$  mixture (pH of 7.4). Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances, including endothelial NO. This avoided possible actions of different drugs on endothelial cells that could lead

to misinterpretation of results. Endothelium was removed by gently rubbing the luminal surface of the segments with a thin wooden stick. The segments were subjected to a tension of 0.5 g, which was readjusted every 15 min during a 90-min equilibration period before drug administration. After this, the vessels were exposed to 75 mmol/L KCl, to check their functional integrity. Endothelium removal did not alter the contractions elicited by 75 mmol/L KCl. After a washout period, the absence of vascular endothelium was tested by the inability of 10  $\mu\text{mol/L}$  acetylcholine (ACh) to relax segments precontracted with NA.

Frequency-response curves to EFS (1, 2, 4, 8 and 16 Hz) were performed. The parameters used for EFS were 200 mA, 0.3 ms, 1-16 Hz, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitisation between consecutive curves. Three successive frequency-response curves separated by 1-hour intervals produced similar contractile responses. To evaluate the neural origin of the EFS-induced contractile response, the nerve impulse propagation blocker, tetrodotoxin, (TTX, 0.1  $\mu\text{mol/L}$ ) was added to the bath 30 min in advance.

To determine the participation of adrenergic innervations in the EFS-induced response in segments from control and  $\text{HgCl}_2$ -treated rats, 1  $\mu\text{mol/L}$  phentolamine, an  $\alpha$ -adrenoceptor antagonist, was added to the bath 30 min before performing the frequency-response curve. Additionally, the vasoconstrictor response of exogenous NA (1 nmol/L-10  $\mu\text{mol/L}$ ) was tested in segments from both experimental groups.

To analyse the participation of NO in the EFS-induced response in segments from both groups of rats, 0.1 mmol/L  $\text{N}^\omega$ -nitro-L-arginine methyl ester (L-NAME), a non-specific inhibitor of nitric oxide synthase (NOS), was added to the bath 30 min before performing the second frequency-response curve. To rule out the inducible origin of NO, a similar protocol was made with the specific inducible NOS inhibitor or 1400W (1  $\mu\text{mol/L}$ ). The vasodilator response to the NO donor, diethylamine NONOate, (DEA-NO, 0.1 nmol/L-0.1 mmol/L) was determined in NA-precontracted arteries from both experimental groups. Participation of superoxide anions ( $\text{O}_2^-$ ) on the vasodilator response to DEA-NO was tested by incubating an  $\text{O}_2^-$  scavenger with 0.1 mmol/L tempol.

To study the possible participation of sensitive innervation on EFS-induced response in segments from control and  $\text{HgCl}_2$ -treated rats, 0.5  $\mu\text{mol/L}$  8-37 CGRP, a CGRP receptor antagonist, was added to the bath 30 min before performing the second frequency-response curve. The vasodilator response to exogenous CGRP (0.1 nmol/L-0.1  $\mu\text{mol/L}$ ) was analysed in NA-precontracted segments from both groups of animals.

#### *Nitric Oxide Release*

NO release was measured using fluorescence emitted by the fluorescent probe 4,5-diaminofluorescein (DAF-2). Endothelium-denuded mesenteric arteries from control and  $\text{HgCl}_2$ -treated rats were subjected to a 60-minute equilibration period in HEPES buffer (in mmol/L: NaCl 119; HEPES 20;  $\text{CaCl}_2$  1.2; KCl 4.6;  $\text{MgSO}_4$  1;  $\text{KH}_2\text{PO}_4$  0.4;  $\text{NaHCO}_3$  5; glucose 5.5;  $\text{Na}_2\text{HPO}_4$  0.15; pH 7.4) at 37 $^\circ$  C. Arteries were incubated with 2  $\mu\text{mol/L}$  DAF-2 for 30 min. The medium was then collected to measure basal NO release. Once the organ bath was refilled, cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals were applied. Afterwards, the medium was collected to measure EFS-induced NO release. The fluorescence of the medium was measured at room temperature using a spectrofluorometer (LS50 Perkin Elmer Instruments, FL

WINLAB Software, Whaltnann, MA, USA) with excitation wavelength set at 492 nm and emission wavelength at 515 nm.

The EFS-induced NO release was calculated by subtracting basal NO release from that evoked by EFS. Also, blank sample measures were collected in the same way from segment-free medium in order to subtract background emission. Some assays were performed in the presence of 0.1 mmol/L L-NAME or 0.1 mmol/L 7-nitroindazol (7NI), the specific nNOS inhibitor, to ensure the specificity of the method. The amount of NO released was expressed as arbitrary units/mg tissue.

#### *Western blot*

For Western blot analysis of nNOS and phosphorylated nNOS (P-nNOS) expression, endothelium-denuded mesenteric segments from both groups of rats were homogenised in a boiling buffer composed of 1 mmol/L sodium vanadate, 1% SDS, and 0.01 M pH 7.4 Tris-HCl. Homogenates containing 30 µg protein were electrophoretically separated on a 7.5% SDS-polyacrylamide gel, and then transferred to polyvinyl difluoride membranes (Bio Rad Immun-Blot) overnight at 4° C, 230 mA, using a Bio-Rad Mini Protean III system (Bio-Rad Laboratories, Hercules, CA, USA) containing 25 mM Tris, 190 mM glycine, 20% methanol, and 0.05% SDS. The membrane was blocked for 1 h at room temperature in Tris-buffered-saline solution (100 mM, 0.9% w/v NaCl, 0.1% SDS) with 5% powdered fat-free milk before being incubated overnight at 4° C with mouse monoclonal antibody against nNOS (1:1000 dilution, Abcam, Cambridge, UK) or against P-nNOS (1:1000, Abcam, Cambridge, UK). After washing, the membrane was incubated with specific horseradish peroxidase-conjugated immunoglobulin G antibody (Amersham International Plc). The membrane was thoroughly washed and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminescence system (ECL Plus, Amersham International Plc, Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, Amersham International Plc, Little Chalfont, UK). Signals on the immunoblot were quantified using a computer program (NIH Image V1.56, National Institute of Health, Bethesda, MD, USA). The same membrane was used to determine α-actin expression, and the content of the latter was used to correct protein expression in each sample by means of a monoclonal antibody anti α-actin (1:2000 dilution, Sigma-Aldrich, Madrid, Spain). Rat brain homogenates were used as a positive control.

#### *Detection of O<sub>2</sub><sup>-</sup>*

O<sub>2</sub><sup>-</sup> levels were measured using lucigenin chemiluminescence. Mesenteric segments were rinsed in KHS for 30 min, equilibrated for 30 min in HEPES buffer at 37° C, transferred to test tubes that contained 1 mL HEPES buffer (pH 7.4) containing lucigenin (5 µmol/L) and then kept at 37° C. The luminometer was set to report arbitrary units of emitted light; repeated measurements were collected for 5 min at 10 s intervals and averaged. 4,5-Dihydroxy-1,3-benzene-disulphonic acid "Tiron" (10 mmol/L), a cell-permeant, non-enzymatic scavenger of O<sub>2</sub><sup>-</sup>, was added to quench the O<sub>2</sub><sup>-</sup>-dependent chemiluminescence. Also, blank measures were collected in the same way without mesenteric segments to subtract background emission.

#### *Noradrenaline release*

NA release was measured using Noradrenaline research EIA (Labor Diagnostica Nord, GmbH and Co., KG, Nordhon, Germany). Endothelium-denuded segments of rat mesenteric arteries from control and HgCl<sub>2</sub>-treated rats were preincubated for 30 minutes in 5 mL of KHS at 37° C and continuously gassed with a 95% O<sub>2</sub>-5% CO<sub>2</sub> mixture (stabilization period). This was followed by two washout periods of 10 min in a bath of 0.4 mL of KHS. Then, the medium was collected to measure basal NA release. Afterwards, the organ bath was refilled, and cumulative EFS periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals were applied. Afterwards, the medium was collected to measure EFS-induced NA release. The assay was performed following the manufacturer's instructions. Results were expressed as ng NA/mL mg tissue.

#### *Drugs used*

L-NA hydrochloride, ACh chloride, diethylamine NONOate diethylammonium salt, 8-37 CGRP, rat CGRP, TTX, L-NAME hydrochloride, 7-nitroindazol, 1400W, phentolamine, tempol, lucigenin, tiron and DAF-2 (Sigma-Aldrich, Madrid, Spain) were used. Stock solutions (10 mmol/L) of drugs were made in distilled water, except for NA, which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w/v) solution. These solutions were kept at -20 °C and appropriate dilutions were made in KHS on the day of the experiment.

#### *Data Analysis*

The responses elicited by EFS and NA were expressed as a percentage of the initial contraction elicited by 75 mmol/L KCl for comparison between control and HgCl<sub>2</sub>-treated rats. The relaxation induced by DEA-NO and CGRP was expressed as a percentage of the initial contraction elicited by NA (Control: 995.43 ± 2.3 mg; HgCl<sub>2</sub>: 986.91 ± 4.6 mg; P > 0.05). Results are given as mean ± SEM. Statistical analysis was done by comparing the curve obtained in the presence of the different substances with the previous or control curve by means of repeated measure analysis of variance (ANOVA), using the GraphPad Prism 5.0 software (CA, USA). Some results were expressed as differences of area under the curve (dAUC) of DEA-NO obtained in segments from control and HgCl<sub>2</sub>-treated animals. AUC were calculated from the individual concentration-response curves/plot and the differences were expressed as a percentage of the difference in AUC of the corresponding control situation. For dAUC, NO, CGRP and NA release data, the statistical analysis was done using Student's t test for unpaired experiments. P < 0.05 was considered significant.



## Results

No differences in body weight were observed between control and HgCl<sub>2</sub>-treated rats either before or after treatment [Control: before 293 ± 6.2 g and after 375 ± 8.9 g (n=10); HgCl<sub>2</sub>: before 287 ± 4.8 g and after 366 ± 6.1 g (n=10)]. Blood pressure was not modified by HgCl<sub>2</sub> treatment (Control: 133 ± 11.4 mm Hg; HgCl<sub>2</sub>: 126 ± 8.4 mm Hg; P > 0.05; n = 10 animals each group). Rats exposed to 30 days mercuric chloride treatment presented a whole blood mercury level of 7.97 ± 0.59 ng/ml. A previous study in untreated comparable male Wistar rats was unable to detect blood Hg [37].

### *Vascular responses to EFS*

In endothelium-denuded mesenteric arteries the response induced by 75 mmol/L KCl was similar in segments from both groups of rats (control, 1300 ± 79.5 mg, HgCl<sub>2</sub>, 1400 ± 75.1 mg; P > 0.05; n = 10 each group). However, the contractions induced by EFS were higher in mesenteric segments from HgCl<sub>2</sub>-treated rats (Figure 1). EFS-induced contractions were practically abolished in segments from both experimental groups by the nerve impulse propagation blocker, TTX (0.1 μmol/L) (Results not shown).

### *Effect of HgCl<sub>2</sub> treatment on adrenergic component of vascular responses to EFS*

Basal NA release was higher in mesenteric segments from HgCl<sub>2</sub>-treated than from control rats. (Figure 2A). EFS increased NA release in both groups of rats, but to a greater extent in segments from HgCl<sub>2</sub>-treated rats (Figure 2A). The contractile response induced by exogenous NA (0.1 nmol/L–10 mmol/L) was greater in segments from HgCl<sub>2</sub>-treated than from control rats (Figure 2B). In agreement with these results, the α-adrenoceptor antagonist, phentolamine (1 μmol/L), significantly decreased the EFS-induced contraction in segments from both groups (Figure 2C and 2D), but this decrease was greater in segments from HgCl<sub>2</sub>-treated rats. (Table 1).

### *Effect of HgCl<sub>2</sub> treatment on nitergic component of vascular responses to EFS*

Basal NO release was higher in mesenteric segments from control than from HgCl<sub>2</sub>-treated rats (Figure 3A). EFS increased NO release in both groups of rats, but to a greater extent in control rats (Figure 3A). Preincubation with L-NAME (0.1 mmol/L) or 7NI (0.1 mmol/L) abolished NO release in arteries from both groups (Results not shown). nNOS expression was similar in homogenates from both experimental groups (Figure 3B), while p-nNOS expression was diminished in segments from HgCl<sub>2</sub>-treated rats (Figure 3B).

Preincubation with non-specific NOS inhibitor (0.1 mmol/L) increased EFS-induced contraction in segments from both groups of rats. This increase was greater in segments from control than from HgCl<sub>2</sub>-treated rats (Figure 3C and 3D, Table 1). Preincubation with the specific iNOS inhibitor 1400W (1 μmol/L) did not modify EFS-induced contraction in either group of rats (Figure 3C and 3D).

In segments precontracted with NA, the NO donor, DEA-NO, (0.1 nmol/L–0.1 mmol/L) induced a concentration-dependent relaxation, which was greater in segments from control than from HgCl<sub>2</sub>-treated rats (Figure 4A, Table 2). Preincubation with 0.1 mmol/L tempol increased the vasorelaxation induced by DEA-NO in both experimental

groups, but this increase was greater in segments from HgCl<sub>2</sub>-treated animals (Figure 4B and 4C, table 2). In agreement with this result, after subtracting the lucigenin chemiluminescence obtained in the presence of tiron from the chemiluminescence obtained in its absence, O<sub>2</sub><sup>-</sup> production was significantly higher in segments from HgCl<sub>2</sub>-treated rats than in segments from control rats. (Figure 4D).

#### *Effect of HgCl<sub>2</sub> treatment on sensory component of vascular responses to EFS*

The CGRP receptor antagonist 8-37 CGRP (0.5 μmol/L) did not modify the contractile response induced by EFS in any experimental group (Results not shown). Additionally, in segments precontracted with NA, CGRP (0.1 nmol/L–1 μmol/L) induced a concentration-dependent relaxation, which was similar in segments from both experimental groups (Results not shown).

### **Discussion**

According to the United States Environmental Protection Agency, the recommended adverse reference blood concentration of mercury is 5.8 ng/mL. Below this concentration level, exposure is considered to not have adverse effects [38, 39]. Several studies have shown that the blood mercury level in the control population is 1 ng/mL. However, residents and mercury exposed workers from Guizhou, China, which is a region known to have mercury contamination, had levels ranging from 7 to 10 ng/mL [40, 41]. In a recent biomonitoring study carried out in New York City adults, the blood concentration of mercury was 2.73 ng/mL, reaching 5.65 ng/mL in regular fish consumers [42]. In the present study, we used low doses of mercuric chloride to treat rats and attained a blood mercury content of around 8 ng/mL at the end of treatment, close to the human exposed levels reached. In previous studies we have demonstrated that HgCl<sub>2</sub> treatment is a risk factor for cardiovascular disease, since it produces alterations in endothelial function in both conductance and resistance arteries, including coronary arteries [19, 43]. Endothelial dysfunction is produced by HgCl<sub>2</sub>, as a result of the decreased NO bioavailability induced by increases in O<sub>2</sub><sup>-</sup> production [25]. Additionally, we have reported that the vasoconstrictor response induced by α-adrenoceptor activation is also increased [19, 25]. However, vascular tone control can be also altered by modifications in vascular innervation. The present study shows for the first time that in mesenteric arteries from HgCl<sub>2</sub>-treated rats the vasomotor response induced by EFS is increased, suggesting changes in innervation function. Increased neuronal NO metabolism produced by an increased superoxide generation and increased NA release participates in this effect. Therefore, results regarding the chronic treatment with HgCl<sub>2</sub> obtained by our group up until now indicate that this metal alters vasomotor tone control by altering the function mediated by endothelium and the innervation of vessels.

Vascular tone in mesenteric arteries is modulated by adrenergic, nitrergic and sensory innervations [26, 27]. As previously described [31], the application of EFS to mesenteric arteries induces vasoconstrictor responses that are abolished by TTX, indicating that in our experimental conditions the vasomotor response induced by EFS has a neuronal origin. It has been reported that mercury affects neurotransmitter secretion from neuronal ending tissue [33, 34]. In this work we have found that treating rats with HgCl<sub>2</sub> increased vasoconstrictor responses to EFS in superior mesenteric arteries. EFS produced a vasoconstrictor response, which is the integrated result of

released neurotransmitters, mainly NA, NO and CGRP [31, 44-46]. In addition, the role of adrenergic, nitrgergic and sensory innervations may change depending on the physiopathological conditions [30, 47, 48].

Since adrenergic innervation plays an important role in the vasoconstrictor response induced by EFS in mesenteric artery [27], we evaluated the possible effect of HgCl<sub>2</sub> treatment on adrenergic function. Phentolamine decreased EFS-induced contractile response in both experimental groups but the decrease was more marked in segments from mercury-treated rats, indicating an increased role of adrenergic innervation in HgCl<sub>2</sub>-treated animals. This effect could be due to increased NA release and/or alterations in the response of vascular smooth muscle cells to NA. The results obtained in this work indicate that the increased response to EFS observed in mesenteric arteries from HgCl<sub>2</sub>-treated rats is mediated at least in part by increased NA release from adrenergic nerve endings. These results agree with previous reports of increased NA release induced by mercury in rat hippocampus [33, 49], even though a diminished NA uptake cannot be ruled out, as has been previously described [50]. Mercury-induced inhibition of Na<sup>+</sup>K<sup>+</sup>-ATPase (NKA) leads to an increased intracellular Na<sup>+</sup> concentration [10], ultimately increasing intracellular Ca<sup>2+</sup>. This mechanism might participate in the increased NA release observed in our study. Additionally, our results show that in segments from HgCl<sub>2</sub>-treated animals, vasoconstrictor response to NA was higher than in segments from control animals. These results agree with previous reports from our group, where phenylephrine response was increased in aorta, caudal and mesenteric resistance arteries from HgCl<sub>2</sub>-treated rats [19, 25].

The NO released from nitrgergic innervation has been demonstrated to have a vasodilator role contributing to the vasomotor response [31]. It has been reported that mercury inhibits NO production and NOS activity in cultured human umbilical vascular endothelial cells [51] and that NO bioavailability is decreased, induced by increases in oxidative stress through increased activity of NADPH oxidase [25]. Previously, we have demonstrated that both unspecific NOS inhibitor L-NAME and specific nNOS inhibitor 7-NI, decrease EFS-induced NO release to a similar extent [52]. However, in vascular reactivity experiments, preincubation with 7-NI decreases vasoconstrictor response to NA, leading to a decrease in EFS-induced contractions [52, 53]. For that reason, we used the unspecific NOS inhibitor L-NAME in our vascular reactivity experiments. EFS-induced vasoconstriction was increased in both experimental groups by preincubation with L-NAME. However, this increase was lower in the HgCl<sub>2</sub>-treated group compared to controls suggesting that chronic HgCl<sub>2</sub> treatment reduced the nitrgergic innervation function. The reduction of nitrgergic function in the HgCl<sub>2</sub>-treated group could be the result of a diminished NO release and/or altered metabolism. The present study shows that NO release was reduced in the HgCl<sub>2</sub>-treated group compared to controls, which would explain the lower participation of NO in vasomotor response to EFS. Neuronal NO production in neural tissue has two sources, nNOS and iNOS [52-54]. The specific iNOS inhibitor 1400W did not change the vasoconstrictor response to EFS in segments from either experimental group, ruling out the participation of this inducible isoform and suggesting that NO release was, indeed, dependent on nNOS expression and/or activity. In previous reports we have reported both no modification or an increase on eNOS expression induced by HgCl<sub>2</sub> treatment, depending on the vascular bed analysed [19]. Variations in differential expression of nNOS and eNOS depending on the vascular tissue analysed and physiopathological conditions have been reported [55]. In this sense, we have previously observed in hypertension that the increase in nNOS expression [56] is not accompanied by changes in eNOS expression [57]. In this study, we found that nNOS protein expression was not modified by the mercury

treatment, while P-nNOS expression was decreased. In previously reported data, eNOS expression was not altered in aorta segments, but was increased in segments from mesenteric resistance arteries [19]. These results were obtained in different vascular beds, and a decrease on eNOS activity was only suggested [19]. However, in this study we have demonstrated a real decrease on nNOS activity by measuring P-nNOS expression. This lower nNOS activity would explain the decreased NO release. However, we cannot rule out diminished NO bioavailability. This possibility is based on previous findings showing that  $\text{HgCl}_2$  induces superoxide production [25], which metabolizes NO and additionally produces peroxynitrite, which has a vasoconstrictor action [58]. Two experimental protocols were performed to clarify this issue. Preincubation with the superoxide anion scavenger, tempol, increased vasodilation induced by NO donor DEA-NO in both experimental groups, but the increase was greater in segments from  $\text{HgCl}_2$ -treated animals. We applied a similar NA precontraction to mesenteric segments from both experimental groups by using cumulative NA doses, thus avoiding the possible differences in vasodilator response to DEA-NO that would have appeared if different precontraction amounts were given. Additionally, superoxide anion production was also measured and a large increase was found. These findings demonstrate that chronic treatment with  $\text{HgCl}_2$  reduces neuronal NO bioavailability through reduced nNOS activity and enhanced superoxide anion production, thus increasing the NO metabolism.

We have previously reported that in this strain sensory innervation does not participate in vasomotor response induced by EFS [31]. However, we have observed that sensory innervation might increase its participation in several pathological conditions such as hypertension and diabetes [28, 29]. Indeed, the participation of peptidergic innervation was not observed in any of the experimental groups when 8-37 CGRP was used, suggesting that  $\text{HgCl}_2$  treatment did not affect this innervation.

In conclusion, these results indicate that chronic treatment with low doses of  $\text{HgCl}_2$  produces an increased vasoconstriction in response to EFS in rat mesenteric arteries. This effect is mediated at least in part by alterations to adrenergic and nitrergic function but not through the sensory innervation. In nitrergic innervation  $\text{HgCl}_2$  actions are a result of reduced NO release and bioavailability, probably caused by reduced nNOS activity and increased superoxide anion production. The increased adrenergic function is caused by increased NA release and vasoconstrictor response. Low-dose mercury exposure is more common than expected, with unclear health consequences. The present findings show that chronic low doses of mercury have an important and deleterious effect on vascular function. However, whether this effect increases the consequences of traditional risk factors or whether it plays a primary role as a cardiovascular risk factor still needs to be determined.

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### Author contribution

Javier Blanco-Rivero performed the experiments, analysed the data, discussed the results and wrote the manuscript; Lorena B. Furieri performed part of the experiments; Dalton V. Vassallo was involved in discussing the results; and Mercedes Salaiques and Gloria Balfagón were involved in discussing the results and writing the manuscript.

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Tables

**Table 1: EFS inhibition/potential after preincubation with 1  $\mu\text{mol/L}$  phentolamine or 0.1 mmol/L L-NAME, respectively.**

	1 Hz	2 Hz	4 Hz	8 Hz	16 Hz
<b>Control + phentolamine</b>	100.3 $\pm$ 10.1	97.4 $\pm$ 12.3	84.6 $\pm$ 9.5	62.5 $\pm$ 4.3	29.2 $\pm$ 5.1
<b>HgCl<sub>2</sub> + phentolamine</b>	100.4 $\pm$ 12.3	99.9 $\pm$ 11.8	96.3 $\pm$ 8.5	94.9 $\pm$ 6.1*	85.4 $\pm$ 7.9*
<b>Control+ L-NAME</b>	95.1 $\pm$ 20.3	76.2 $\pm$ 15.4	95.5 $\pm$ 35.3	105.6 $\pm$ 19.8	78.6 $\pm$ 22.3
<b>HgCl<sub>2</sub> + L-NAME</b>	17.5 $\pm$ 5.3*	46.3 $\pm$ 9.5*	39.4 $\pm$ 8.3*	35.9 $\pm$ 10.4*	25.4 $\pm$ 12.3

Percentage (%) of Inhibition/potential on EFS-induced contraction after preincubation with 1  $\mu\text{mol/L}$  phentolamine or 0.1 mmol/L L-NAME in control rats, and 30 day HgCl<sub>2</sub>-treated rats. Calculations are performed taking control EFS-induced contraction as 100% of the contractile response. Results are expressed as mean  $\pm$  SEM. \*P<0.05 vs. control.

**Table 2: E<sub>max</sub> and log EC<sub>50</sub> values of vasodilator responses to DEA-NO in mesenteric arteries from control and HgCl<sub>2</sub> treated rats before and after preincubation with tempol (0.1 mmol/L)**

	Control		HgCl <sub>2</sub>	
	E <sub>max</sub>	log EC <sub>50</sub>	E <sub>max</sub>	log EC <sub>50</sub>
<b>Untreated</b>	97.53 $\pm$ 1.49	- 6.90 $\pm$ 0.04	91.08 $\pm$ 2.35*	- 6.19 $\pm$ 0.11*
<b>Tempol-treated</b>	102.70 $\pm$ 4.54	- 7.38 $\pm$ 0.16 <sup>#</sup>	99.20 $\pm$ 2.61 <sup>#</sup>	- 7.13 $\pm$ 0.15 <sup>#</sup>

Results are expressed as means  $\pm$  S.E.M. of the number of animals indicated between brackets. \*P < 0.05 HgCl<sub>2</sub>-treated vs. untreated animals. # P < 0.05 tempol incubated vs. unincubated segments.

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**Figure Legends:**

**Figure 1: Vasoconstrictor response to EFS in segments from control and HgCl<sub>2</sub>-treated rats.** Results (Mean  $\pm$  S.E.M.) were expressed as a percentage of the initial contraction elicited by KCl. \*P < 0.05 vs. control animals for each frequency (Bonferroni test). n=10 animals each group.

**Figure 2: Effect of HgCl<sub>2</sub> treatment on adrenergic innervation.** (A) Basal and EFS-induced NA release in mesenteric segments from control and HgCl<sub>2</sub>-treated rats. Results (Mean  $\pm$  S.E.M.) are expressed as ng NA/mL mg tissue. \* P < 0.05 vs. basal; # P < 0.05 vs. control. n= 6 animals per group. (B) Vasoconstrictor response to exogenous NA in segments from control and HgCl<sub>2</sub>-treated rats. Results (Mean  $\pm$  S.E.M.) are expressed as a percentage of the previous contraction elicited by KCl. n=8 animals each group. Effect of preincubation with 1  $\mu$ mol/L phentolamine on the vasoconstrictor response induced by EFS in mesenteric segments from control (C) and HgCl<sub>2</sub>-treated rats (D). Results (Mean  $\pm$  S.E.M.) are expressed as a percentage of the previous contraction elicited by KCl. n=6 animals each group. \*P < 0.05 vs. conditions without specific inhibitor for each frequency (Bonferroni test).

**Figure 3: Effect of HgCl<sub>2</sub> treatment on nitrenergic innervation.** (A) Basal and EFS-induced NO release in segments from control and HgCl<sub>2</sub>-treated rats. Results (Mean  $\pm$  S.E.M.) are expressed as arbitrary units (A.U.)/ mg tissue. \* P < 0.05 vs. basal; # P < 0.05 vs. control. HFD. n= 4 animals per group. (B) Effect of HgCl<sub>2</sub> treatment on nNOS and P-nNOS expression. The blot is representative of four separate segments from each group. Lower panel shows relation between P-nNOS and nNOS expression. Results (Mean  $\pm$  S.E.M.) are expressed as the ratio of the signal obtained for P-nNOS and that obtained for nNOS. \* P < 0.05 vs. control. Effect of preincubation with 0.1 mmol/L L-NAME on the vasoconstrictor response induced by EFS in mesenteric segments from control (C) and HgCl<sub>2</sub>-treated rats (D). Results (Mean  $\pm$  S.E.M.) are expressed as a percentage of the previous contraction elicited by KCl. n= 8 animals each group. \*P < 0.05 vs. conditions without specific inhibitor for each frequency (Bonferroni test).

**Figure 4: Effect of HgCl<sub>2</sub> treatment on vasodilator response to DEA-NO.** (A) Vasodilator response to NO donor DEA-NO in segments from control and HgCl<sub>2</sub>-treated rats. Effect of preincubation with 0.1 mmol/L tempol on the vasodilator response elicited by DEA-NO in segments from control (B) and HgCl<sub>2</sub>-treated animals (C). Results (Mean  $\pm$  S.E.M.) are expressed as a percentage of the initial contraction elicited by exogenous NA. \*P < 0.05 vs. control conditions for each dose (Bonferroni test). n= 7 animals each group. The attached graph shows the dAUC to DEA-NO for segments in the absence or presence of 0.1 mmol/L tempol. dAUC values are expressed as a percentage of the difference of the corresponding AUC for the segments in the absence of tempol (\*P < 0.05). (D) Superoxide anion release in mesenteric segments from control and HgCl<sub>2</sub>-treated rats. Results (Mean  $\pm$  S.E.M.) are expressed as chemiluminescence units (U)/min mg tissue. \* P < 0.05 vs. control. n= 4 animals per group.

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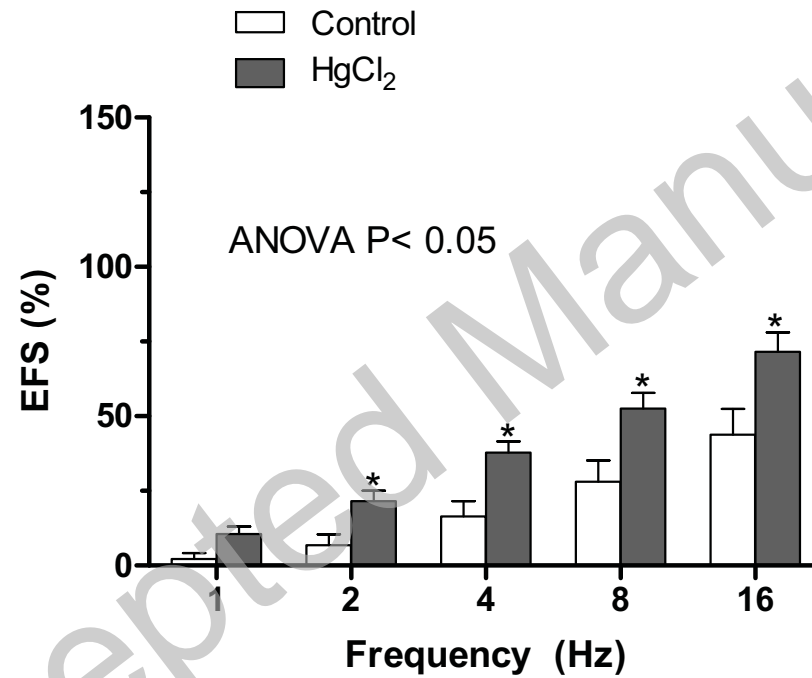


Figure 1

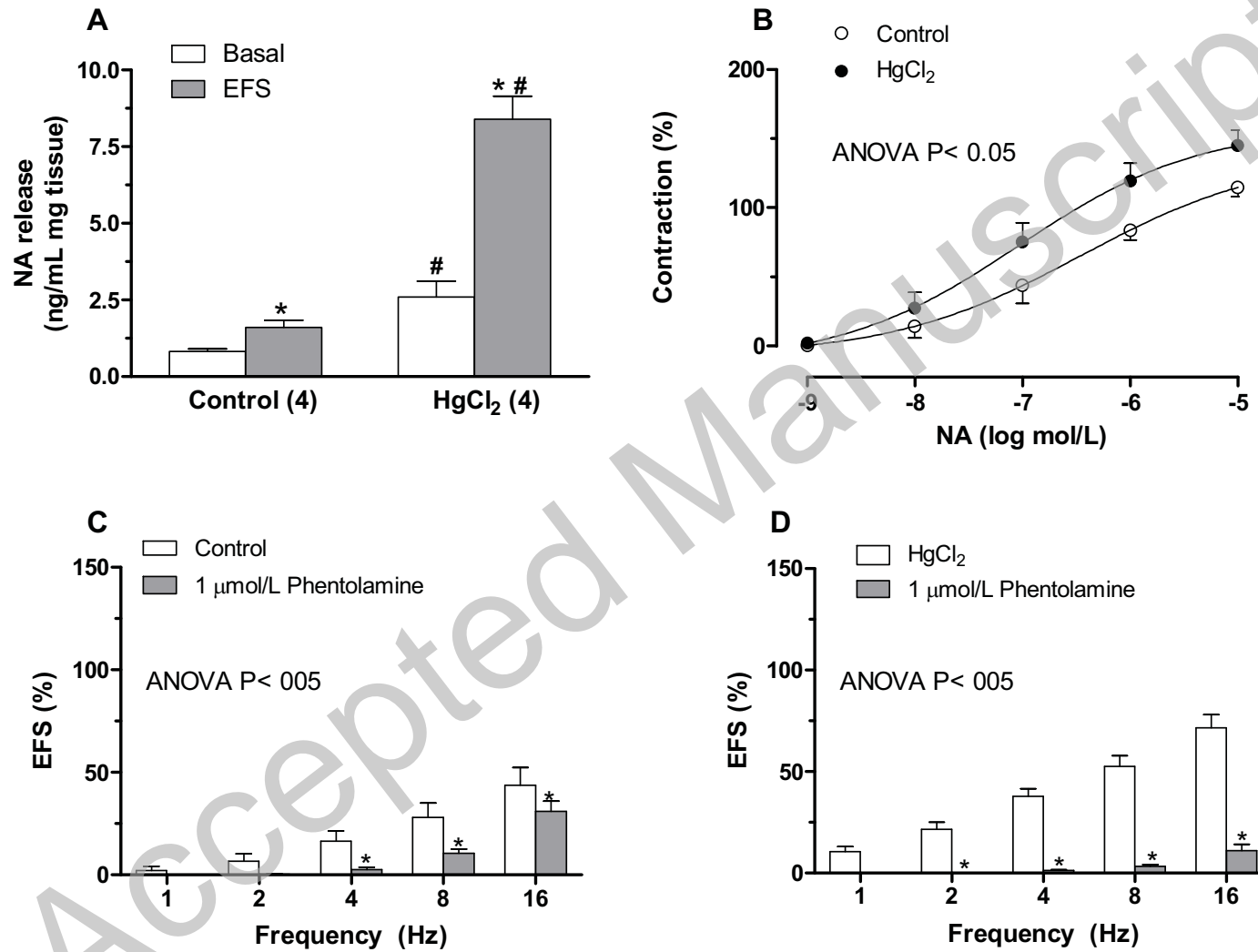


Figure 2

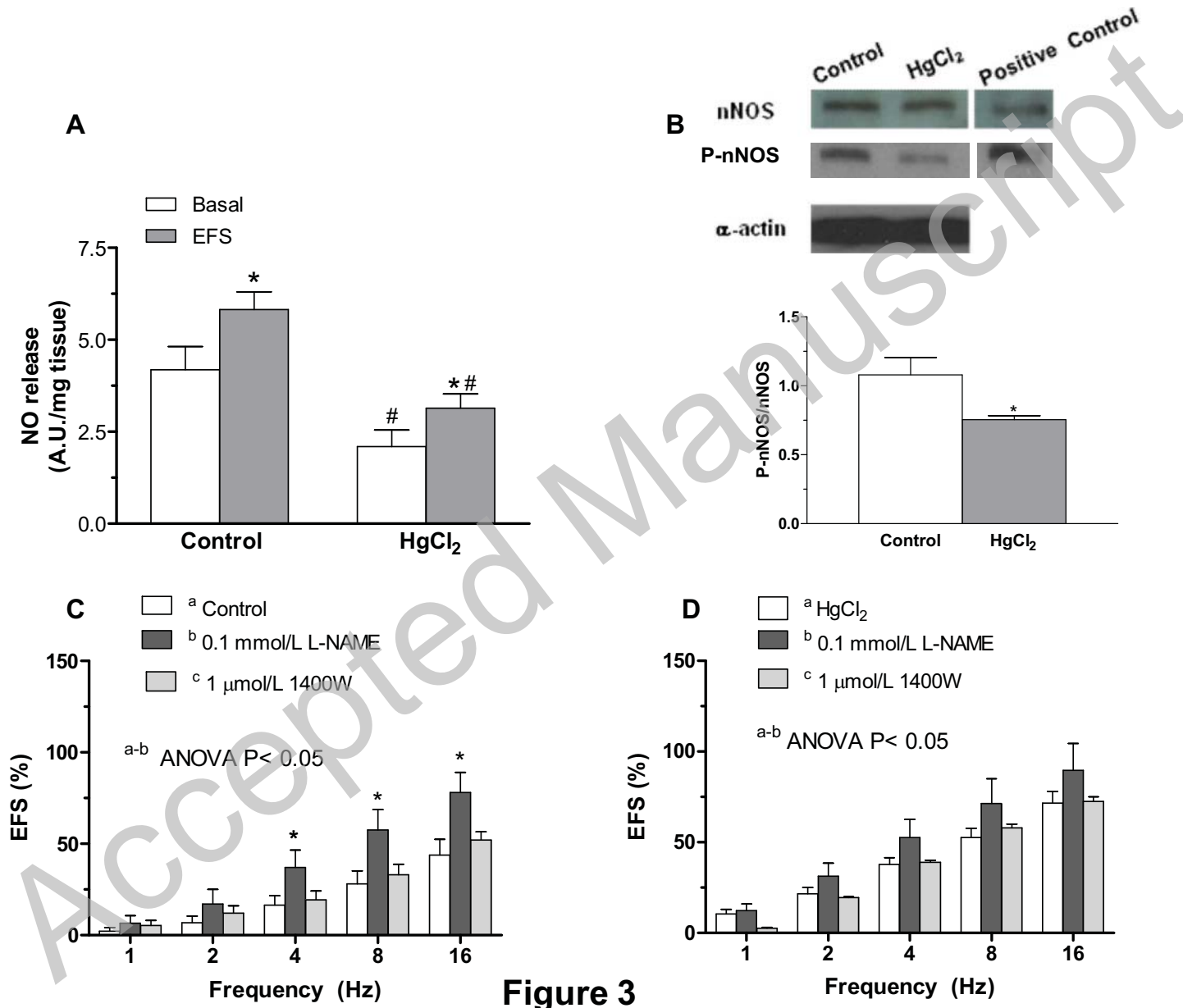


Figure 3

