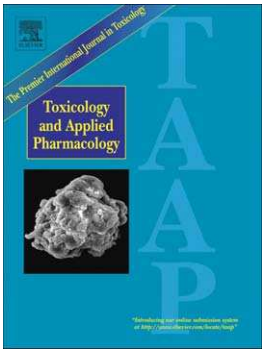


Exposure to low mercury concentration *in vivo* impairs myocardial contractile function

Lorena Barros Furieri, Mirian Fioresi, Rogério Faustino Ribeiro Junior, María Visitación Bartolomé, Aurélia Araújo Fernandes, Victoria Cachofeiro, Vicente Lahera, Mercedes Salaices, Ivanita Stefanon, Dalton Valentim Vassallo

PII: S0041-008X(11)00237-7
DOI: doi: 10.1016/j.taap.2011.06.015
Reference: YTAAP 12138

To appear in: *Toxicology and Applied Pharmacology*

Received date: 7 March 2011
Revised date: 6 June 2011
Accepted date: 10 June 2011

Please cite this article as: Furieri, Lorena Barros, Fioresi, Mirian, Junior, Rogério Faustino Ribeiro, Bartolomé, María Visitación, Fernandes, Aurélia Araújo, Cachofeiro, Victoria, Lahera, Vicente, Salaices, Mercedes, Stefanon, Ivanita, Vassallo, Dalton Valentim, Exposure to low mercury concentration *in vivo* impairs myocardial contractile function, *Toxicology and Applied Pharmacology* (2011), doi: 10.1016/j.taap.2011.06.015

Exposure to low mercury concentration *in vivo* impairs myocardial contractile function

[1]Lorena Barros Furieri, [1]Mirian Fioresi, [1]Rogério Faustino Ribeiro Junior, [4]María Visitación Bartolomé, [1]Aurélia Araújo Fernandes, [4]Victoria Cachofeiro, [4]Vicente Lahera, [3]Mercedes Salaices, [1]Ivanita Stefanon, [1,2] Dalton Valentim Vassallo.

[1]Department of Physiological Sciences, Federal University of Espirito Santo, Vitoria, ES, Brazil

[2]Health Science Center of Vitoria- EMESCAM, Vitoria, ES, Brazil.

[3]Department of Pharmacology, Universidad Autónoma de Madrid, Spain

[4]Department of Physiology, Universidad Complutense de Madrid, Spain

Correspondence to: Dalton V. Vassallo

Department of Physiological Sciences,

Federal University of Espirito Santo,

Av. Marechal Campos, 1468 Maruípe

29040-091, Vitoria, ES, Brasil.

FAX: +55-27-2122 7330.

1 e-mail: daltonv2@terra.com.br

2

Running head: Hg treatment and myocardial contractility dysfunction

ABSTRACT

Increased cardiovascular risk after mercury exposure has been described but cardiac effects resulting from controlled chronic treatment are not yet well explored. We analyzed the effects of chronic exposure to low mercury concentrations on hemodynamic and ventricular function of isolated hearts.

Wistar rats were treated with $HgCl_2$ (1st dose 4.6 µg/kg, subsequent dose 0.07 µg/kg/day, *im,* 30 days) or vehicle. Mercury treatment did not affect blood pressure (BP) nor produced cardiac hypertrophy or changes of myocyte morphometry and collagen content. This treatment: 1) *in vivo* increased left ventricle end diastolic pressure (LVEDP) without changing left ventricular systolic pressure (LVSP) and heart rate; 2) in isolated hearts reduced LV isovolumic systolic pressure and time derivatives, and β-adrenergic response; 3) increased myosin ATPase activity; 4) reduced Na^+-K^+ ATPase (NKA) activity; 5) reduced protein expression of SERCA and phosphorylated phospholamban on serine 16 while phospholamban expression increased; as a consequence SERCA/phospholamban ratio reduced; 6) reduced sodium/calcium exchanger (NCX) protein expression and α-1 isoform of NKA, whereas α-2 isoform of NKA did not change.

Chronic exposure for 30 days to low concentrations of mercury does not change BP, heart rate or LVSP but produces small but significant increase of LVEDP. However, in isolated hearts mercury treatment promoted contractility dysfunction as a result of the decreased NKA activity, reduction of NCX and SERCA and increased PLB protein expression. These findings offer further evidence that mercury chronic exposure, even at small concentrations, is an environmental risk factor affecting heart function.

Keywords: Mercury, Na^+-K^+ ATPase, contractility dysfunction, SERCA/PLB ratio.

Introduction

Adverse effects of mercury were mainly recognized in the 1970s due to the disasters of Minamata Bay (Eto 2000) and Iraq (Bakir et al., 1973). Increased cardiovascular risk after mercury exposure has been reported, and both acute and chronic mercury exposure produces several toxic effects on the cardiovascular system.

Acute mercury administration reduces arterial blood pressure (Rhee and Choi, 1989; Rossoni et al., 1999b) and myocardial contractility (Oliveira et al., 1994). Acute $HgCl_2$ (5 mg/Kg) also produces cardiac systolic and diastolic failure, and pulmonary hypertension *in vivo* (Rossoni et al., 1999b). In left ventricular papillary muscles, 0.5 and 1 μM $HgCl_2$ increase force development (Oliveira et al., 1994; Assis et al., 2003) probably resulting from the inhibition of sarcolemmal Na^+,K^+-ATPase (NKA) (Anner et al., 1992). At higher concentrations, mercury produces a negative inotropism as a consequence of calcium overload by reducing sarcoplasmic reticulum Ca^{2+}-ATPase activity (Hechtenberg and Beyersmann, 1991). The metal also reduces tetanic tension development and myosin ATPase activity (Vassallo et al., 1999; Moreira et al., 2003) at these concentrations. In Langendorff-perfused hearts, perfusion with high concentrations of mercury also reduces cardiac contractility, thereby decreasing isovolumic pressure development (Rhee and Choi, 1989, Massaroni et al., 1995).

Attention has recently been focused on the cardiovascular toxic effects of chronic mercury exposure and its association with hypertension, carotid atherosclerosis, myocardial infarction and coronary heart disease (Salonen et al., 2000; Virtanen et al., 2005; Houston, 2007). Different forms of mercury, such as $HgCl_2$ and methyl mercury, have different actions and adverse outcomes when acutely or when higher doses are used. For chronic low dose exposure the proximate toxic agent is most likely inorganic mercury (Rooney, 2007). Moreover, studies reporting mercury effects resulting from chronic exposition are still scarce and the underlying mechanisms are not yet well explored.

In order to adequately control amounts of mercury absorption, we developed an experimental model for controlled chronic exposure to low concentrations of $HgCl_2$; such a model describes an endothelial dysfunction in aorta and mesenteric resistance arteries due to decreased NO bioavailability by

increased NADPH oxidase-derived O_2^- (Wiggers et al., 2008). We then investigated whether the effects of chronic exposure to low concentrations of mercury also affects cardiac contractility by evaluating effects on arterial and ventricular pressures, isolated heart, NKA and myosin ATPase activities, expression of calcium handling proteins and changes in myocyte morphometry. Findings provide further evidence that chronic exposure to low doses of mercury, even at concentrations considered to be safe, is an environmental risk factor for heart function and cardiovascular disease.

Material and Methods

Animals. Three-month-old male Wistar rats were obtained from the Animal Quarters of the Federal University of Espirito Santo. 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 standard chow *ad libitum*. Rats were divided into two groups: control (vehicle – saline solution, *im*) and those treated with mercury chloride for 30 days (1st dose 4.6 µg/kg, subsequent dose 0.07 µg/kg/day, *i.m* to cover daily loss). Our group described that this treatment led to blood levels of ~ 8 ng/mL (Wiggers et al., 2008). All experiments were conducted in compliance with the guidelines for biomedical research as stated by the Brazilian Societies of Experimental Biology, were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by local ethics comitte (CEUA-EMESCAM 003/2007, 007/2007).

Arterial blood pressure, ventricular pressure and heart rate measurements. At the end of treatment, rats (N= 22) were anesthetized with urethane (1.2 g/kg, Sigma (St Louis, MO, USA), and a polyethylene catheter (PE50) filled with heparinized saline (50 U/ml) was introduced into the carotid artery to measure arterial systolic blood pressure (SBP) and diastolic blood pressure (DBP). The carotid artery catheter was introduced into the left ventricle to measure systolic pressure (LVSP) and its positive and negative time derivatives (dP/dt + LV and dP/dt - LV, respectively), as well as left ventricular end diastolic pressure (LVEDP). Recordings were performed over a 30-min period with a pressure transducer (TSD104A), and with an interface and data collection software (MP100A, BIOPAC System, Inc., Santa Barbara, CA, USA). Heart rate (HR) was determined from intra-beat intervals.

Isolated heart perfusion. After treatment, rats (N= 14) were anesthetized with urethane (1.2 g/kg), treated with heparin (500 UI, *i.p.*) and sacrificed by exsanguination; the heart was excised after 10 minutes and mounted in an isolated organ chamber and perfused according to the Langendorff technique at constant flow (10 mL/min) with Krebs-Henseleit bicarbonate buffered solution containing

(in mM): 120 NaCl, 5.4 KCl, 1.25 $CaCl_2$, 2.5 $MgSO_4$, 1.2 Na_2SO_4, 2.0 NaH_2PO_4, 20 $NaHCO_3$, and 11 glucose (salts used were of analytical grade; Sigma, St Louis, MO, USA and Merk, Darmstat, Germany). This solution was filtered and continuously bubbled with 95% O_2 and 5% CO_2 (pH=7.4) and kept between 34-35 ºC. After mounting, the left atrium was opened and a soft distensible balloon mounted at the tip of a rigid plastic tube was inserted into the left ventricular cavity through the atrioventricular valve. To avoid liquid accumulation in the ventricular cavity, the ventricle was perforated with a puncture needle. The balloon was connected, via a Y piece, to a pressure transducer (TSD 104A) and to a syringe so that the diastolic pressure of the left ventricle could be adjusted to predetermined values by injecting water into the balloon. The resulting pressure was registered. The hearts were driven with isolated suprathreshold rectangular pulses (5 ms duration) at a constant rate (3.3 Hz) through a pair of Ag/AgCl electrodes attached to the upper region of the right ventricle. Mechanical activity was investigated by measuring developed left ventricular isovolumic systolic pressure (LVISP). To evaluate contractility the rate of rise of LVISP (dP/dt) was used because it is highly sensitive to changes in contractility (Gleason and Braunwald, 1962). These parameters were measured with a pressure transducer connected to an amplifier (MP 100 Biopac Systems: Inc.; CA) and recorded with a data acquisition system (BIOPAC MP100WSW, including a software Acqknowledge III, Goleta, CA). The isovolumic pressure derivative (dP/dt) was gotten offline by the same software (digital filter Blackman -61dB, 25 KHz of cut frequency and sample rate of 1000/s). All measurements began 30 minutes after mounting to allow the beating preparation to adapt to the *in vitro* conditions. The coronary perfusion pressure (CPP) was continuously registered by connecting a pressure transducer (TDS 104A) to the inflow of the aortic pressure tube. Since coronary flow was kept constant (10 mL/min), changes of the CPP were dependent on changes of coronary resistance.

Protocols were performed beginning with a constant diastolic pressure of 5 mmHg by adjusting the volume of the balloon. Ventricular function curves were obtained by measuring the left ventricular isovolumic systolic pressure (LVISP) developed while diastolic pressure was increased from 0 to 30 mmHg in steps of 5 mmHg. Balloon volume was kept constant during experiments involving other protocols; this permitted changes in diastolic and systolic pressures to be measured. Initially, recordings were taken under control conditions in both groups. In order to analyze inotropic response, a single dose of isoproterenol (Sigma, St Louis, MO, USA) *in bolus* (100 µl, 10 µM) was administered to evaluate β-adrenoceptor response.

Assay of Cardiac Myosin ATPase Activity. Some animals were killed at the end of hemodynamic measurements. The hearts were rapidly frozen in liquid nitrogen and kept at -80ºC until the day of analysis. Briefly, as previously reported (Moreira et al., 2003), myosin was prepared from minced and homogenized left ventricles extracted with KCl-phosphate buffer (0.3 M KCl and 0.2 M phosphate buffer [pH 6.5](Klotz et al., 1975)). Myosin ATPase activity was assayed according to previous reports (Klotz et al., 1975; Cappelli et al., 1989) by measuring inorganic phosphate (Pi) liberation from 1 mM ATP in the presence of 50 mM HEPES (pH 7), 0.6 M KCl, 5 mM $CaCl_2$, or 10 mM ethylene glycol-bis (β-amino ethyl ether)-N,N,N',N'-tetra acetic acid (EGTA) in a final volume of 200 µL. Samples were assayed in duplicate or triplicate and corrected for non-enzymatic hydrolysis by using controls assayed in the same conditions, except that the protein sample was added after the interruption of the reaction by using 200 µL of 10 % trichloroacetic acid. The reaction was initiated by addition of the protein sample to avoid the inactivation at 30 ºC caused by a lack of substrate. The enzyme activity was calculated as the difference between activities observed in the presence of Ca^{2+} and that in the presence of 10 mM EGTA. Pi was determined by the method of Chan *et al* (1986) (Chan et al., 1986). The specific activity was reported as nmol Pi released per min per mg of protein. Protein was measured by the Coomassie blue method using bovine serum albumin as a standard (Bradford, 1976).

Na^+-K^+ ATPase activity assay. The enzymatic material was extracted as described by Velema and Zaagsma (1981) with the following modifications: ventricular tissue was homogenized in a solution containing Tris-HCl 20 mM and EDTA 1 mM pH 7.5. Na^+-K^+ ATPase activity was assayed by measuring Pi liberation from 3 mM ATP in the presence of NaCl 125 mM, $MgCl_2$ 3 mM, KCl 20 mM and Tris-HCl 50 mM (pH 7.5). The enzyme was preincubated for 5 minutes at 37ºC and the reaction was initiated by adding the ATP. Incubation times and protein concentration were chosen in order to ensure the linearity of the reaction. The reaction was stopped by the addition of 200 µL of 10 % trichloroacetic acid. Controls with addition of the enzyme preparation after addition of trichloroacetic acid were used to correct for nonenzymatic hydrolysis of the substrate. All samples were in duplicate. The specific activity was reported as nmol Pi released per min per mg of protein unless otherwise stated. The specific activity of enzyme was determined in the presence and absence of 5 mM of ouabain.

Western blot analysis. After Langendorff experiments, hearts were homogenized and proteins [50 µg for PLB, PLB- phospho-Ser^{16} and 100 µg for SERCA, NCX, α-1, α-2] were separated by 7.5%

(SERCA, NCX, α-1 and α-2), 15% (PLB) SDS-PAGE. Proteins were transferred to nitrocellulose membranes and were incubated with mouse monoclonal antibodies for SERCA (1:500, Affinity BioReagents, CO, USA), NCX (1:200, Abcam Cambridge, MA, USA), PLB (2 μg/mL, Affinity BioReagents, CO, USA), α-1 (1:1000, Upstate, Billerica, MA) or rabbit polyclonal antibodies for PLB phospho-Ser16 (1:5000, Badrilla, Leeds, UK) and α-2 (1:1000, Upstate, Billerica, MA). After washing, membranes were incubated with anti-mouse or anti-rabbit (1:5000, StressGen, Victoria, Canada) immunoglobulin antibody conjugated to horseradish peroxidase. After thorough washing, immunocomplexes were detected using an enhanced horseradish peroxidase/luminal chemiluminescence system (ECL Plus, Amersham International, Little Chalfont, UK) and film (Hyperfilm ECL International). Signals on the immunoblot were quantified with the National Institutes of Health Image V1.56 computer program. The same membrane was used to determine GAPDH expression using a mouse monoclonal antibody (1:5000, Abcam Cambridge, MA, USA).

Morphometric analysis. In the present study, two different quantifications were considered in order to analyze putative actions of mercury treatment on cardiac structure. Firstly, a determination was made as to whether treatment could modify the size or morphology of myocyte cell bodies. Secondly, it was determined if the measurements could detect a variation in collagen content.

Cardiomyocyte cell body quantification. Histologic sections of ventricular tissue of 4- μm thickness were stained with Masson's trichrome. Three sections for each animal were analyzed using a high resolution monochromatic photocamera CCD (SonyXC-75CE) attached to a photomicroscope (LeicaDMRB). The morphometric analyses were performed with an Image System Analysis (Leica Q500MC) with 8 bits of images in grey gradation (256 levels of grey: 0 representing black as blank and 255 colors). The binary edition was used to remove artifacts that did not correspond to cardiomyocyte (cell body) stained area. Cardiomyocyte (60-80 per animal) analyses were performed with 40x lenses in which the cell nuclei were clearly observed. The same illumination conditions were used for all measurements. Calibration of the system was carried out using a stage micrometer (Leitz) that allowed computation of the object area in units of μm^2.

Cardiac Collagen Quantification. Regarding the collagen content evaluation we used ***Picrius Sirius*** red staining. This technique is widely used for measuring collagen content in different tissues. Tissue samples were dehydrated, embedded in paraffin and cut in sections of (4 μm) thickness. These sections were stained with 0.5% Sirius Red F3BA (Aldrich Chemical Company). The quantification of collagen in left ventricle was performed using an image analysis system from LEICA (LEICA 500YW,

Cambridge, UK) and expressed as percent of tissue area. A single researcher unaware of the experimental groups performed the analysis.

Data analysis and statistics. Results are reported as means ± SEM. Differences were analyzed using Student's *t*-test or two-way ANOVA followed by a Tukey test. $p \leq 0.05$ was considered significant. For protein expression, data are expressed as the ratio between signals on the immunoblot corresponding to the studied protein and GAPDH.

Results

In rats exposed to 30-day $HgCl_2$ treatment no differences in body weight between groups were observed either before or after treatment. Left and right ventricles weight normalized by body weight also showed no differences (Table 1).

In order to investigate cardiac effects, Langendorff-perfused hearts from both controls and from 30-day exposed rats were used. Figure 1 shows that the LVISP was reduced in the mercury-treated group with diastolic pressure fixed at 5 mmHg. Reduction was also observed for positive and negative dP/dt, while coronary perfusion pressure did not change. When performing ventricular function curves, LVISP was reduced in the mercury-treated group for all diastolic pressure values (Figure 2A). Similar behavior was obtained with positive and negative dP/dt (Figure 2B-C), but diastolic pressure increments did not change the coronary perfusion pressure (data not shown).

Isoproterenol was used in order to test if $HgCl_2$ treatment could alter the myocardial response to inotropic interventions. Isoproterenol administration (100 μL, 10 μM, *in bolus*) increased LVISP and positive and negative dP/dt in both groups. However, the increase in LVISP and positive dP/dt showed a percentage reduction in the mercury-treated group suggesting a reduction in the β-adrenergic inotropic action (Figure 3). During this inotropic intervention no changes in coronary perfusion pressure were observed.

In order to investigate the underlying mechanisms that could explain those mechanical effects on hearts from mercury-treated rats, NKA and myosin ATPase activities were measured. Myocardial myosin ATPase activity was increased (Ct: 257.7 ± 11.7 vs $HgCl_2$: 301.5 ± 9,3 nmol Pi/min/mg, $P<0.05$) while NKA activity was reduced (Ct: 59.8 ± 4.5 vs $HgCl_2$: 35.3 ± 8.6 nmol Pi/min/mg, $P<0.05$) in the mercury-treated group. Protein expressions of NCX, α1 and α2 subunits of NKA, SERCA, phospholamban and its phosphorilated fraction were measured (Figures 4 and 5) in order to evaluate

calcium handling mechanisms. After 30-day mercury treatment reduces expression of alfa-1 NKA subunit and NCX expression, but expression of alfa-2 NKA subunit did not change. Moreover, after exposure to low doses of mercury, SERCA expression and phosphorilated phospholamban were reduced while phospholamban expression was increased. These changes led to a reduction in SERCA/PLB ratio, suggesting the reduction in calcium uptake by the sarcoplasmic reticulum contributing to the calcium overload.

Since no changes in myocardial mass were found analyzing ventricular weight we investigated myocardial morphology by analyzing putative actions of mercury treatment on cell morphometry. No changes for morphometric results were observed for perimeter, width, length and area of myocardial cells (results not shown). In addition, no changes were observed in collagen fraction in left ventricle from the mercury-treated group as compared with controls (5.1 ± 0.8 *vs* 4.7 ± 0.6 % of tissue area).

To investigate if such changes could produce hemodynamic effects, we performed measurements of systolic, diastolic, mean arterial pressure and heart rate in anesthetized rats. No differences were observed between the two groups (Table 2). The measurement of ventricular pressures, LVSP and time derivatives also did not change. However, only LVEDP increased in the 30-day mercury-treated group.

Discussion

The main findings presented here show that 30-day treatment with low mercury concentration produced a negative inotropic effect in perfused hearts, although myosin ATPase activity increased. This contractility reduction was explained by alterations in calcium-handling mechanisms because of both diminished protein expression of SERCA, NKA (α1 subunit) and NCX, and increased PLB expression together with a reduced response to β-adrenergic stimulation. This treatment did not change arterial or ventricular pressures, although it produced a small but significant increase of LVEDP in anesthetized rats.

Mercury is known to be an environmental risk factor for cardiovascular diseases (Houston, 2007). The reference blood concentration of mercury recommended by the US Enviromental Protection Agency's, below which exposure is considered to be without adverse effect, is 5.8 ng/mL (National Academy of Science, 2000; Rice, 2005; Stern, 2005). In control populations, blood mercury concentration was reported to be 2.73 ng/mL in adults in New York City and 1 ng/mL in China. Mercury attains levels of 5.65 ng/mL (McKelvey et al., 2007) in regular fish consumption, and in workers that are regularly exposed attain levels between 7 to 10 ng/mL (Gupta et al., 1996; Chen et al., 2005).

Professional exposure to mercury vapor and release of mercury from removal of amalgam dental fillings increases its blood (18 nM; ~5 ng/mL) and plasma (5 nM; ~1.5 ng/mL) concentration (Halbach, 1995; Bjorkman et al., 1997; Langworth et al., 1997; Sandborgh-Englund et al., 1998). This form of the exposure is represented by elemental mercury. Once absorved it can be oxidized into inorganic mercury (Rooney, 2007; Björkman et al., 2007). Professional exposure also produces central nervous system alteration (Langworth et al., 1997) and tooth fillings impair kidney function (Carmignani et al., 1989).

Mercury exerts its effects by combining with SH groups (Clarkson, 1972) and these actions might be the manner by which the metal exerts its effects on the cardiac myocytes (Halbach, 1989). However, the fact

that mercury can concentrate inside the cells suggests that the metal might produce effects at even lower concentrations under chronic exposure. We have previously reported that chronic exposure to small concentrations do produce harmful vascular effects (Furieri et al., 2011; Wiggers, 2008) but studies regarding cardiac function with chronic exposure to low concentrations of the metal are scarce.

Given that relatively high blood levels of mercury are more likely to pose the metal as an environmental risk factor for cardiovascular diseases, we recently developed a method for producing a controlled chronic mercury administration that attains a blood concentration of 8 ng/mL (~29 nM). Using a similar exposure protocol, and to avoid effects of humoral and neural factors that exist in the blood, the perfused heart preparation was used. In these preparations a negative inotropic and lusitropic effect was observed in the mercury-treated group. The underlying mechanisms that could explain these findings are usually the reduction of NKA, NCX and SERCA activities (Bers, 2006). Biochemical analyses performed here showed that chronic mercury treatment reduces NKA activity, the expression of alfa-1 NKA subunit and NCX expression. Results also show reduction of SERCA expression, PLB increase and phosphorilated PLB reduction, which might lead to less calcium uptake and release. Such condition is known to reduce force development (Bers and Despa, 2006).

Chronic mercury treatment reduces NKA activity leading to increased intracellular sodium concentration, which reduces NCX activity, producing a calcium overload condition. In addition, the reduction of SERCA expression and phosphorilated phospholamban, while occurring an increase in phospholamban expression, reduced the SERCA/PLB ratio. These changes suggest that calcium uptake by the sarcoplasmic reticulum is reduced, which contributes to the calcium overload. Consequently, the release of less calcium upon activation reduces force development.

Taken together these findings explain the reduced mechanical activity found in isolated hearts suggesting that mercury treatment might produce calcium overload. Considering that for the isolated perfused heart there is no protection by homeostatic mechanisms, the perfused heart showed reduction of cardiac mechanical activity, reinforcing the suggestion that this treatment begins to present signs of Hg toxicity.

Although mercury treatment reduced pressure generation, coronary perfusion pressure remained unchanged, even when isoproterenol was used. β-adrenergic activation should produce a vasodilatation after pressure increment. However, we should emphasize that the coronary flow depends mainly on a metabolic control (Gutterman and Cowley Jr, 2006). Considering that both control and Hg-treated

hearts presented similar coronary perfusion pressure we concluded that the coronary flow used was sufficient to maintain myocardial metabolic demands.

Since signs of mercury toxicity were observed *in vitro* we investigated mercury effects in anesthetized animals. Arterial and ventricular pressures were measured. No changes were observed compared to the non-treated rats. Similar results were found for arterial systolic pressure measured in awake rats when using a tail cuff technique (Wiggers et al, 2008). We should consider that the *in vitro* assay is not a good model to reproduce what occurs *in vivo.* A possible explanation for why *in vitro* LVISP was reduced in mercury-treated perfused hearts is the increased myosin ATPase activity and a putative rise in sympathetic tone that reduced the β-adrenergic response to isoproterenol found in the isolated perfused heart.

However, the increment of LVEDP and reduction in dP/dt during relaxation observed in mercury-treated rats indicate that there is some damage to ventricular mechanisms. We observed a reduction of NKA activity, NCX and SERCA expression and an increase in PLB expression. These findings taken together explain the generation of a calcium overload condition and LVEDP increase after mercury treatment. What is more, SERCA activity reductions and PLB increases are usually accompanied by increased LVEDP (Sjaastad et al., 2003), which is not unlike what is observed in other conditions such as heart failure. This negative inotropism and lusitropism *in vivo* were then blunted by the increased myosin ATPase activity and a rise in sympathetic tone. It is worth emphasizing that β-adrenergic activation regulates myosin ATPase activity through cyclic adenosine monophosphate, which explains this association (Winegrad et al., 1986).

Because LVW/BW and RVW/BW did not change we also performed morphometric analyses. After mercury treatment no changes were observed for perimeter, length, width or area of myocytes. Regarding the evaluation in collagen content in mercury-treated animals compared with controls no changes were observed. Since 30 days mercury treatment with low doses did not produce morphological alterations our findings suggest that functional changes here described are not consequence of morphological changes.

Potential limitations of the study. In the present study, we used fluid-filled manometric system as a method for performing the hemodynamic experiments. If we compared the present results with those performed using microtip pressure transducers, we observed that the present values obtained with

polyethylene catheter are lower when compared to those obtained with the microtip catheter (Zimmer and Millar, 1998). Results using the microtip catheter are commonly performed in anesthetized rats, thereby reducing differences with the fluid-filled catheters. Because the use of anesthesia changes hemodynamic parameters, we used the fluid-filled manometric system to perform the present experiments, keeping in mind both the catheter's resonance effect and dumping which this manometric system produces. In any case, as the same fluid-filled manometric system was used to perform all experiments, we believe the present results to be acceptable.

In summary, results presented herein suggest that controlled chronic exposure to small concentrations of inorganic mercury, leading to plasma levels similar to those found after continuous occupational exposure, begins to affect heart function, eventhough several cardiovascular parameters, such as arterial pressure and LVSP measured *in vivo*, are still within normal ranges. In perfused hearts, however, a negative inotropic effect was found resulting from reduction in NKA activity, NCX and SERCA expression and PLB increases, together with a percentage reduction in the magnitude of the β-adrenergic response. It is important to emphasize that, although functional changes are not showing differences *in vivo,* heart function is maintained by compensatory or adaptive mechanisms such as sympathetic activation and increased myosin ATPase activity. These results reinforce the relevance of human chronic occupational exposure to small mercury concentration as a risk factor for heart function.

Acknowledgements

This study was supported by grants from "Ministerio de Ciencia e Innovación" (MCINN) (SAF 2009-07201),"Instituto de Salud Carlos III" ISCIII (Red RECAVA, RD06/0014/0011 and RD06/0014/0007) and Banco Santander Central Hispano, Spain, and by grants from "Coordenação de Aperfeiçoamento de pessoal de Nível superior" (CAPES), "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq), "Fundação de Amparo à Pesquisa do Espírito Santo" (FAPES) and "Fundo Estadual de Ciência e Tecnologia" (FUNCITEC- 39767531/07), Brazil.

Conflicts of Interest Statement

None declared

REFERENCES

Anner, B.M., Moosmayer, M., Imesch, E., 1992. Mercury blocks the Na-K-ATPase by a ligand-dependent and reversible mechanism. Am. J. Physiol. 262, F830-F836.

Aschner, M., Aschner, J.L., 1990. Mercury neurotoxicity, Mechanisms of blood-brain barrier transport. Neurosci. Biobehav. Rev. 14, 169-176.

Assis, G.P.S., Silva, C.E., Stefanon, I., Vassallo, D.V., 2003. Effects of small concentrations of mercury on the contractile activity of the rat ventricular myocardium. Comp. Biochem. Physiol. C 134, 375-383.

Bakir, F., Damluji, S.F., Amin-Zaqi, L., Murtadha, M., Khalidi, A., al-Rawi, N.Y., Tikriti S, Dahahir HI, Clarkson TW, Smith JC, Doherty RA., 1973. Methylmercury poisoning in Iraq. Science 181, 230-241.

Bers, D.M., 2006. Altered Cardiac Myocyte Ca Regulation In Heart Failure. Physiology 21, 380-387.

Bers, D.M., Despa, S., 2006. Cardiac myocytes Ca^{2+} and Na^{+} regulation in normal and failing hearts. J. Pharmacol. Sci. 100, 315-322.

Björkman, L., Sandborgh-Englund, G., Ekstrand, J., 1997. Mercury in saliva and feces after removal of amalgam fillings. Toxicol. Appl. Pharmacol. 144,156-162.

Björkman, L., Lundekvam B.F., Laegreid, T., Bertelsen, B.I., Morild, I., Lilleng, P., Lind, B., Palm, B., Vahter, M., 2007. Mercury in human brain, blood, muscle and toenails in relation to exposure: an autopsy study. Environ. Health 11, 6-30.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 218-254.

Cappelli, V., Bottinelli, R., Poggesi, C., Moggio, R., Reggiani, C., 1989. Shortening velocity and myosin and myofibrillar ATPase activity related to myosin isoenzyme composition during postnatal development in rat myocardium. Circ. Res. 65, 446-457.

Carmignani, M., Boscolo, P., Preziosi, P., 1989. Renal ultraestructural Alterations and Cardiovascular Changes in Rats Exposed to Mercuric Chloride. Arch. Toxicol. 13, 353-356.

Chan, K., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca^{2+}-ATPase activity. Anal. Biochem. 157, 375-380.

Chen, C., Qu, L., Li, B., Xing, L., Jia, G., Wang, T., Gao, Y., Zhang, P., Li, M., Chen, W., Chai, Z., 2005. Increased oxidative DNA damage, as assessed by urinary 8-hydroxy-2-deoxyguanosine concentrations, and serum redox status in persons exposed to mercury. Clin. Chem. 51, 759–767.

Clarkson, T.W., 1972. The biological properties and distribution of mercury. Biochem. J. 130, 61-63.

Eto, K., 2000. Minamata disease. Neuropathology 20, 14-19.

Furieri, L.B., Galán, M., Avendaño, M.S., García-Redondo, A.B., Aguado, A., Martínez, S., Cachofeiro, V., Bartolomé, M.V., Alonso, M.J., Vassallo, D.V., Salaices, M., 2011. Endothelial dysfunction of rat coronary arteries after exposure to low concentrations of mercury is ROS dependent. Br. J. Pharmacol. 162: 1819-1831.

Gleason W.L.; Braunwald, E., 1962. Studies on the first derivative of the ventricular pressure pulse in man. J. Clin. Invest. 41: 80-91.

Gupta, M., Bansal, J.K., Khanna, C.M., 1996. Blood mercury in workers exposed to the preparation of mercury cadmium telluride layers on cadmium telluride base. Ind. Health. 34, 421–425.

Gutterman, D.D., Cowley Jr, A.W., 2006. Relating cardiac performance with oxygen consumption: historical observations continue to spawn scientific discovery. Am. J. Physiol. Heart. Circ. Physiol. 291, 2555-2556.

Halbach, S., 1989. Sulfhydryl-induced restoration of myocardial contractility after alteration by mercury. Arch. Toxicol. 13, 349-352.

Halbach, S., 1995. Combined estimation of mercury species released from amalgam. J. Dent. Res. 74, 1103- 1109.

Hechtenberg, S., Beyersmann, D., 1991. Inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase activity by cadmium, lead and mercury. Enzyme 45, 109-115.

Houston, M.C., 2007. The role of mercury and cadmium heavy metals in vascular disease, hypertension, coronary heart disease, and myocardial infarction. Altern. Ther. Health. Med. 13, 128-133.

Klotz, C., Aumont, M.C., Berson, G., Leger, J.J., Preteseille, M., Swynghedauw, B., 1975. Cardiac myosin, preparation, ATPase in chronic heart hypertrophy. Recent. Adv. Stud. Cardiac. Struct. Metab. 5, 263-271.

Langworth, S., Sallsten, G., Barregard, L., Cynkier, I., Lind, M.L., Soderman, E., 1997. Exposure to mercury vapor and impact on health in the dental profession in Sweden. J. Dent. Res. 76, 1397-1404.

Massaroni, L., Rossoni, L.V., Amaral, S.M.C., Stefanon, I., Oliveira, E.M., Vassallo, D.V., 1995. Hemodynamic and electrophysiological acute toxic effects of mercury in anaesthetized rats and in Langendorff perfused rat hearts. Pharmacol. Res. 32, 27-36.

McKelvey W, Gwyn RC, Jeffry N, Kass D, Thorpe LE, Garg RK, Palmer, C.D., Parsons, P.J., 2007. A biomonitoring study of lead, cadmium, and mercury in the blood of New York City adults. Envirom. Health. Perspect. 115, 1435–1441.

Moreira, C.M., Oliveira, E.M., Bonan, C.D., Sarkis, J.J., Vassallo, D.V., 2003. Effects of mercury on myosin ATPase in the ventricular myocardium of the rat. Comp. Biochem. Physiol., C Toxicol. Pharmacol. 135, 269-275.

National Academy of Sciences. Toxicological Effects of Methylmercury. National Research Council, Washington, DC, USA, 2000.

Oliveira, E.M., Vassallo, D.V., Sarkis, J.J.F., Mill, J.G., 1994. Mercury effects on the contractile activity of isolated heart muscle. Toxicol. Appl. Pharmacol. 128, 86-91.

Rhee, H.M., Choi, B.H., 1989. Hemodynamic and electrophysiological effects of mercury in intact anesthetized rabbits and in isolated perfused hearts. Exp. Mol. Pathol. 50, 281-290.

Rice, D.C., 2005. The US EPA reference dose for methylmercury: sources of uncertainty. Environ. Res. 95, 406-413.

Rooney, JPK., 2007. The role of thiols, dithiols, nutritional factors and interacting ligands in the toxicology of mercury. Toxicology 234, 145-156.

Rossoni, L.V., Amaral, S.M.C., Vassallo, P.F., França, A., Oliveira, E.M., Varner, K.J., Mill, J.G., Vassallo, D.V., 1999b. Effects of mercury on the arterial blood pressure of anesthetized rats. Braz. J. Med. Biol. Res. 32, 989-997.

Salonen, J.T., Seppanen, K., Lakka, T.A., Salonen, R., Kaplan, G.A., 2000. Mercury accumulation and accelerated progression of carotid atherosclerosis: a population-based prospective 4-year follow-up study in men in eastern Finland. Atherosclerosis 148, 265-273.

Sandborgh-Englund, G., Elinder, C.G., Johanson, G., Lind, B., Skare, I., Ekstrand, J., 1998. The absorption, blood levels, and excretion of mercury after a single dose of mercury vapor in humans. Toxicol. Appl. Pharmacol. 150, 146-153.

Sjaastad, I., Wasserstrom, J.A., Sejersted, O.M., 2003. Heart failure -- a challenge to our current concepts of excitation-contraction coupling. J. Physiol. 546, 33-47.

Stern, A.H., 2005. Balancing the risks and benefits of fish consumption. Ann. Intern. Med. 142, 949.

Vassallo, D.V., Moreira, C.M., Oliveira, E.M., Bertollo, D.M., Veloso, T.C., 1999. Effects of mercury on the isolated heart muscle are prevented by DTT and Cysteine. Toxicol. Appl. Pharmacol. 156, 113-118.

Velema, J., Zaagsma, J., 1981. Purification and characterization of cardiac sarcolemma and sarcoplasmic reticulum from rat ventricle muscle. Arch. Biochem. Biophys. 212(2), 678-688.

Virtanen, J.K., Voutilainen, S., Rissanen, T.H., Mursu, J., Tuomainen, T.P., Korhonen, M.J., Valkonen, V.P., Seppänen, K., Laukkanen, J.A., Salonen, J.T., 2005. Mercury, fish oils, and risk of acute coronary events and cardiovascular disease, coronary heart disease, and all-cause mortality in men in eastern Finland. Arterioscler. Thromb. Vasc. Biol. 25, 228-233.

Wiggers, G.A., Peçanha, F.M., Briones, A.M., Pérez-Girón, J.V., Miguel, M., Vassallo, D.V., Cachofeiro, V., Alonso, M.J., Salaices, M., 2008. Low mercury concentrations cause oxidative stress and endothelial dysfunction in conductance and resistance arteries. Am. J. Physiol. Heart. Circ. Physiol. 295, 1033-1043.

Winegrad, S., Weisberg, A., Lin, L.E., McClellan, G., 1986. Adrenergic regulation of myosin adenosine triphosphatase activity. Circ. Res. 58, 83-95.

Zimmer, H.G., Millar, H.D., 1998. Technology and application of ultraminiature catheter pressure transducers. Can. J. Cardiol. 14, 1259-1266.

Figure legends

Figure 1 (A) LVISP- Left ventricle systolic isovolumetric pressure, (B) positive first time derivative (dP/dt +), (C) negative first time derivative (dP/dt -) and (D) coronary perfused pressure from Control (CT) and $HgCl_2$-treated ($HgCl_2$) rats under control conditions in Langendorff apparatus. Results represent mean ± SEM. Number of animals used is indicated in parentheses. *$p<0.05$. Student`s t test.

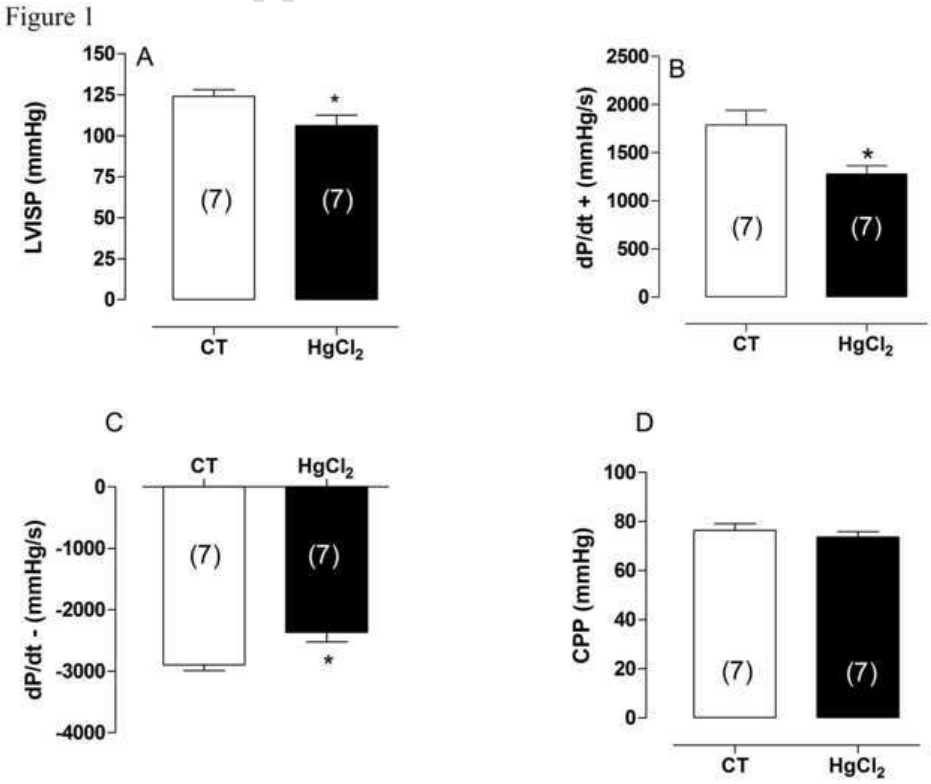


Figure 2 (A) LVISP- Left ventricle systolic isovolumetric pressure, (B) positive first time derivative (dP/dt +), (C) negative first time derivative (dP/dt -) curves obtained at different diastolic pressure (DP) from Control and $HgCl_2$-treated rats. Results represent mean ± SEM. *$p<0.05$. ANOVA two-way and Tukey test.

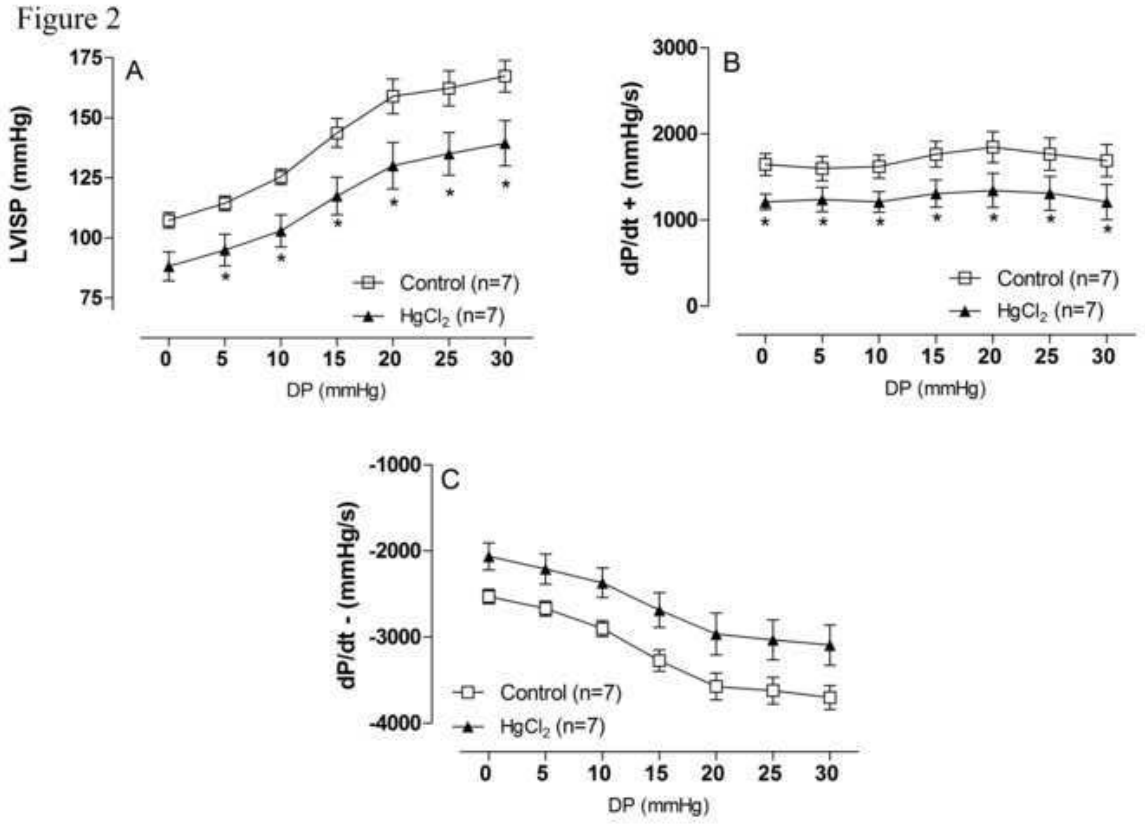


Figure 3 β-adrenergic activation by isoproterenol (100 µL, 10 µM, in *bolus)* in Langendorff apparatus on LVISP (A), dP/dt + (B), dP/dt – (C) and on CPP (D) from Control and HgCl2-treated rats. Results represent mean ± SEM. Number of animals used is indicated in parentheses. *p<0.05. Student`s t test.

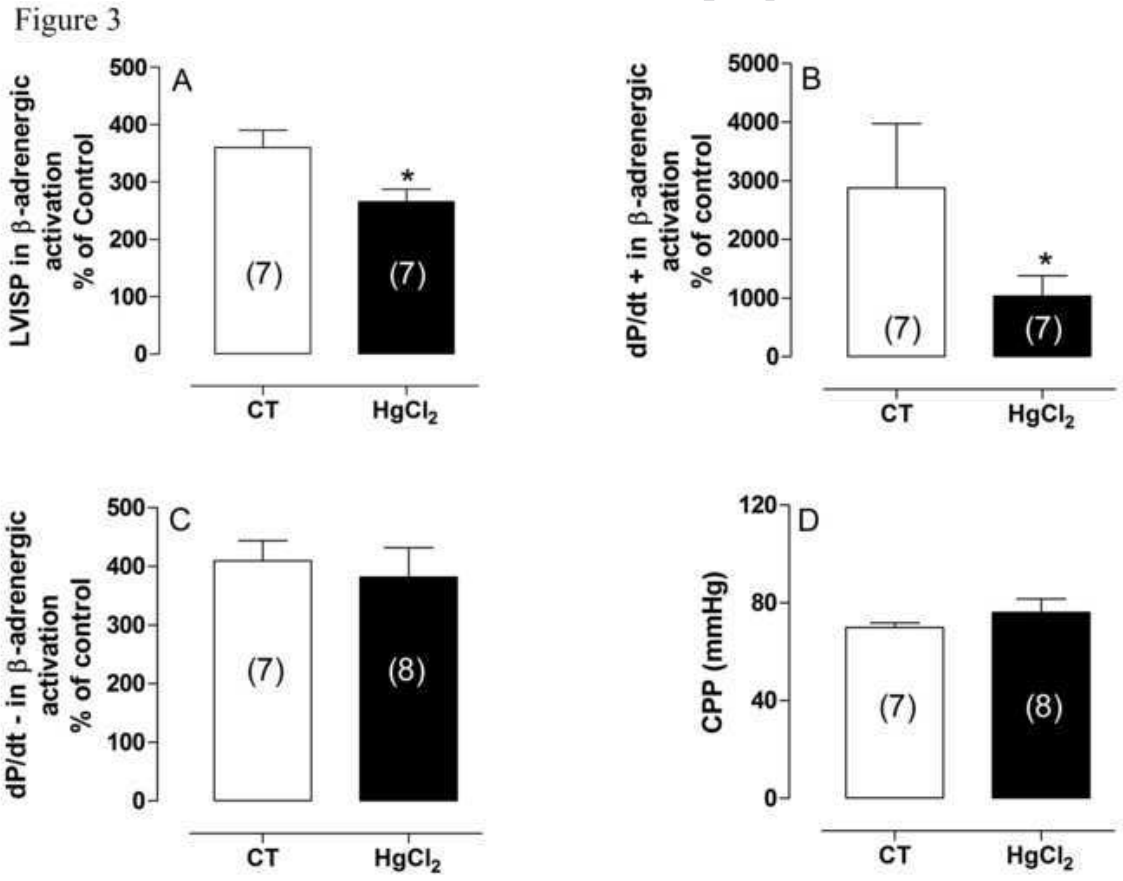


Figure 4 Densitometric analysis of the Western blot for (A) NCX, (B) α-1 and (C) α-2 isoform of Na^+-K^+ ATPase from control and $HgCl_2$-treated rats. Number of animals used is indicated in parentheses. *p<0.05 by Student´s t test. Representative blots are also shown.

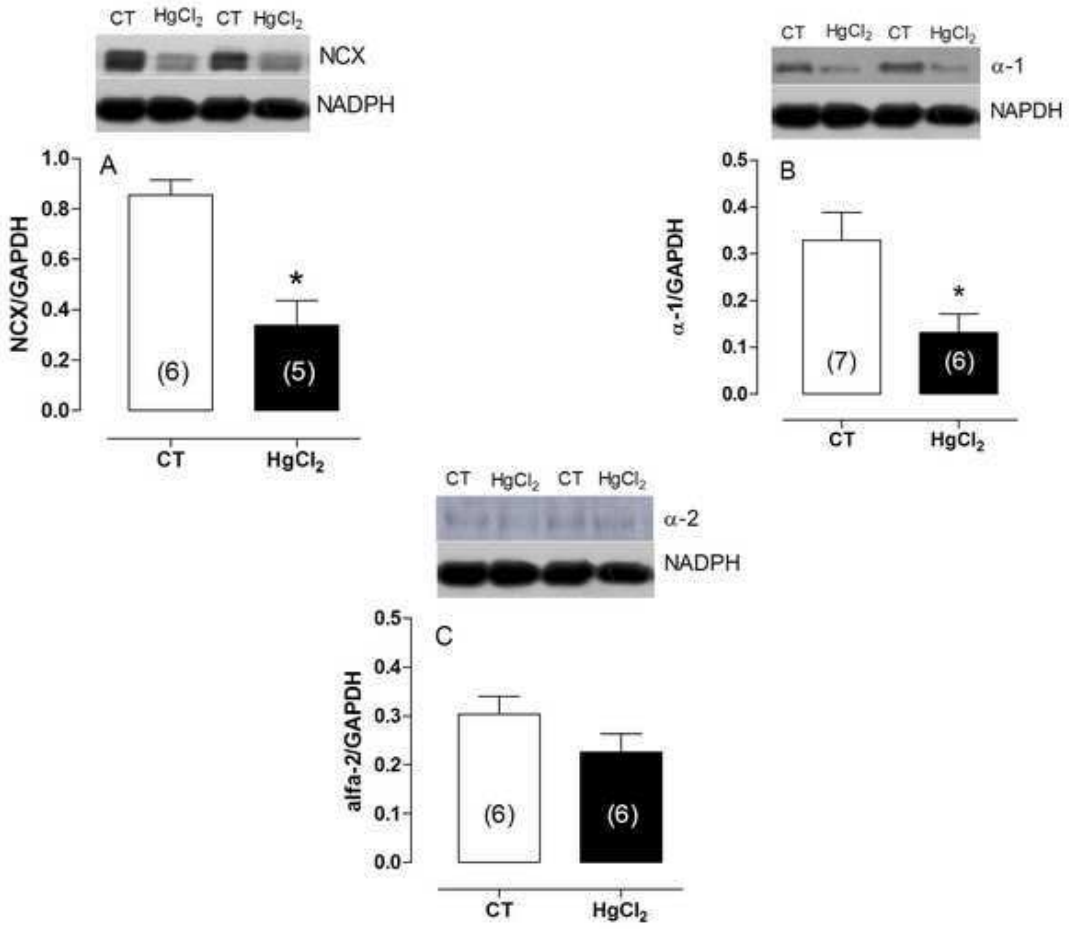
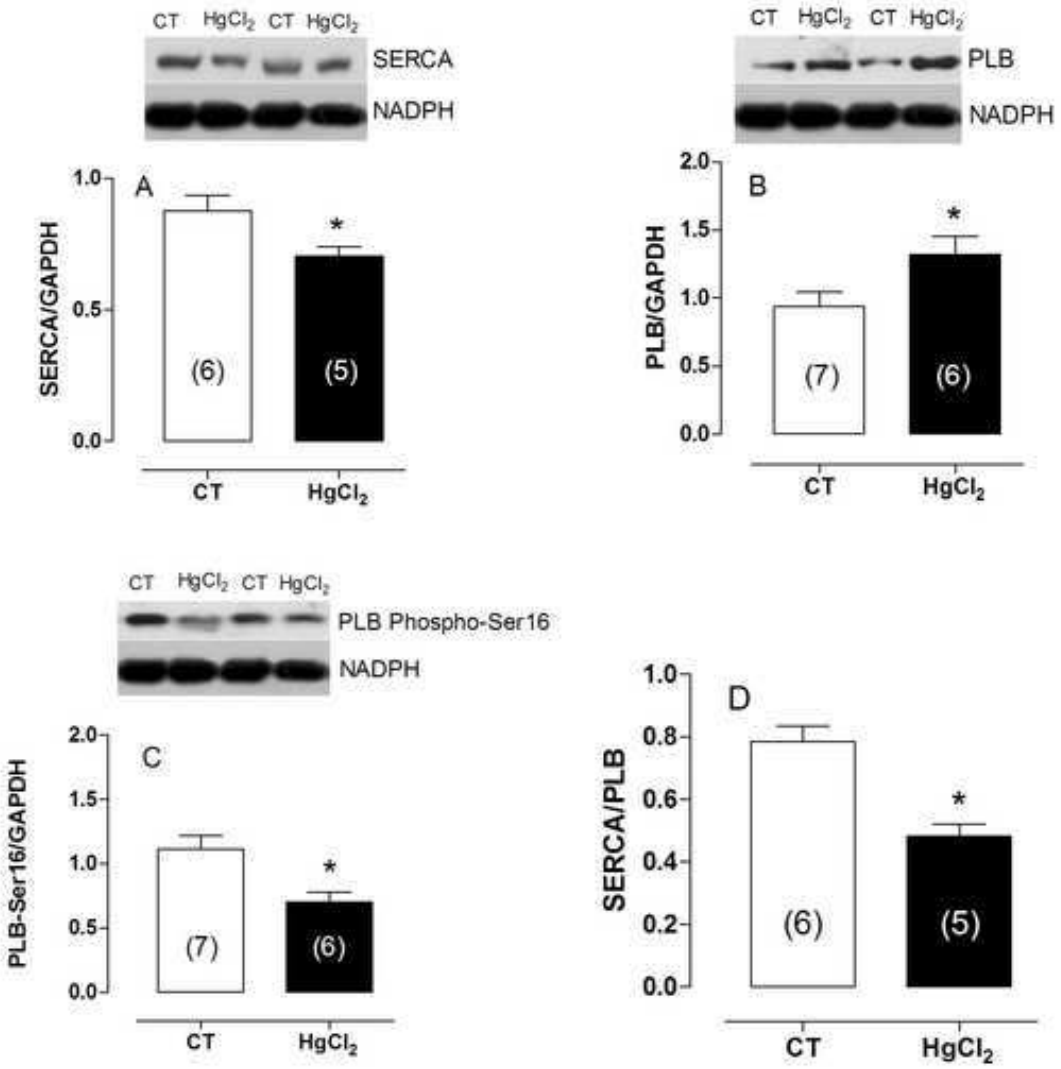


Figure 5 Densitometric analysis of the Western blot for (A) SERCA (B) PLB (C) PLB-phospho Ser[16] and (D) SERCA/PLB ratio protein expression in hearts from control and $HgCl_2$-treated rats. Number of animals used is indicated in parentheses. *p<0.05 by Student´s t test. Representative blots are also shown



Tables

Table 1: Body weight and ventricular weights from Control and $HgCl_2$ treated rats.

	Control	N	$HgCl_2$	N
BW initial (g)	251 ± 4	12	251 ± 5	12
BW final (g)	364 ± 4	12	365 ± 8	12
LV (mg)	886 ± 42	12	904 ± 47	12
LV/BW (mg/g)	2.3 ± 0.1	12	2.4 ± 0.1	12
RV (mg)	229 ± 11	12	227 ± 8	12
RV/BW (mg/g)	0.6 ± 0.02	12	0.6 ± 0.03	12

Initial and final body weight (BW); Left (LV) and right (RV) ventricular weight; LV/BW and RV/BW weight ratios from Control and $HgCl_2$-treated rats. Results represent mean ± SEM. N-Number of animals used. *$p<0.05$. Student`s t test.

Table 2: Hemodynamic parameters from untreated and $HgCl_2$-treated rats.

	Control	N	$HgCl_2$	N
HR (bpm)	303 ± 14	10	319 ± 12	11
SBP (mmHg)	103 ± 6	10	110 ± 5	11
DBP (mmHg)	57 ± 4	10	62 ± 5	11
MBP (mmHg)	77 ± 6	10	75 ± 5	11
LVSP (mmHg)	110 ± 9	8	119 ± 7	10
LVEDP (mmHg)	2.2 ± 0.3	8	3.9 ± 0.4*	10
dP/dt + LV (mmHg/s)	3972 ± 740	8	4579 ± 617	10
dP/dt - LV (mmHg/s)	4827 ± 547	8	4995 ± 495	10

Changes in systolic (SAP), diastolic (DAP) and mean blood pressure(MBP), heart rate (HR), left ventricle systolic pressure (LVSP), left ventricle end diastolic pressure (LVEDP) and in positive (dP/dt

+) and negative first time derivatives (dP/dt -) from Control and $HgCl_2$-treated rats. Results represent mean ± SEM. N- Number of animals used. *$p<0.05$. Student`s t test.

HIGHLIGHTS

This work shows that chronic exposure for 30 days to low concentrations of mercury does not change blood pressure, heart rate or left ventricular systolic pressure but increase end diastolic pressure. In isolated hearts mercury treatment promoted contractility dysfunction as a result of the decreased NKA activity, reduction of NCX and SERCA and increased PLB protein expression. Mercury chronic exposure, even at small concentrations, is an environmental risk factor affecting heart function.