Effects of Chronic Exposure to Mercury on Angiotensin-Converting Enzyme Activity and Oxidative Stress in Normotensive and Hypertensive Rats

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Abstract

Background: Mercury’s deleterious effects are associated with increased cardiovascular risk.

Objective: To determine whether chronic exposure to inorganic mercury increases the activity of angiotensin-converting enzyme and its relationship with oxidative stress in several organs and tissues.

Methods: We studied male Wistar and spontaneously hypertensive rats (SHR) (3-month-old) exposed or not to HgCl₂ for 30 days. At the end of treatment, we investigated the following: changes in body weight, hemodynamic parameters, angiotensin-converting enzyme (ACE) activity and oxidative stress in the heart, aorta, lung, brain and kidney in hypertensive compared to normotensive animals. A value of p < 0.05 was considered significant.

Results: Chronic exposure to HgCl₂ did not affect weight gain in either group. Systolic blood pressure, measured weekly, did not increase in Wistar rats but showed a small increase in SHR rats. We also observed increases in left ventricular end-diastolic pressure and ACE activity in the plasma and hearts of normotensive rats. In the SHR+Hg group, ACE activity increased in plasma but decreased in kidney, lung, brain and aorta. Oxidative stress was assessed indirectly by malondialdehyde (MDA) production, which increased in Hg-treated rats in both plasma and heart. In the SHR+Hg group, MDA increased in heart and aorta and decreased in lungs and brain.

Conclusion: These results suggest that chronic exposure to inorganic mercury aggravates hypertension and produces more expressive changes in ACE activity and oxidative stress in SHRs. Such exposure affects the cardiovascular system, representing a risk factor for the development of cardiovascular disorders in normotensive rats and worsening of pre-existing risks for hypertension. (Arq Bras Cardiol. 2019; 112(4):374-380)

Keywords: Mercury Poisoning; Oxidative Stress/radiation effects; Peptidyl-Dipeptidase A; Hypertension; Rats.

Introduction

Mercury is a toxic metal that causes harmful effects on the cardiovascular system. Blood concentrations levels of 8 ng/mL are found in exposed individuals,1,2 which might have a relationship with hypertension development.3

Several reports showed that mercury induces oxidative stress and might damage several organs and systems.4,5 In addition, increased mercury exposure has been associated with cardiovascular diseases, such as hypertension, carotid atherosclerosis, myocardial infarction and coronary heart disease.10,11 Moreover, oxidative stress is reported to be an efficient mechanism for generation of oxidized low-density lipoprotein and subsequently atherosclerosis;12,13 then, generation of advanced glycation end-products and participation of inflammatory cells take place, sustaining vascular injury.14

One of the main harmful actions of mercury is the generation of oxygen free radicals, NADPH oxidase activation and cyclooxygenase (COX) stimulation induced by mercury may trigger the production of reactive oxygen species (ROS).11,15,16 Moreover, in animal models chronic mercury exposure for 30 days promoted contractility dysfunction in isolated hearts as a result of decreased Na⁺-K⁺-ATPase (NKA) activity, reduction in sodium/calcium exchanger (NCX) and sarco/endoplasmic reticulum calcium ATPase (SERCA) activity and increased phospholamban (PLB) expression.17 Although no effects on blood pressure, heart rate or left ventricular systolic pressure have been reported, mercury causes a small increase in left ventricular end-diastolic pressure in rats.17

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Additionally, at the vascular level, the vasoconstrictor response to phenylephrine was increased in caudal, mesenteric, coronary arteries and in the rat aorta, effects commonly related to reduced bioavailability of nitric oxide (NO) and increased oxidative stress.\textsuperscript{1,18,19} Interacting with NO, superoxide anion (O$\cdot$$_2$) forms peroxynitrite, decreasing NO availability for smooth muscle relaxation.\textsuperscript{20-22}

We reported that mercury administration increases local angiotensin converting enzyme (ACE) activity,\textsuperscript{23} releasing more angiotensin II that enhances the production of reactive radicals.\textsuperscript{23} These results show that mercury pressor effects might depend on angiotensin II generation and are involved in oxidative stress generation. Previous studies showed that mercury could increase local ACE activity and oxidative stress with subsequent oxidative damage in several organs and systems,\textsuperscript{2,17,24-27} but the in vivo effects of mercury chronic exposure on cardiovascular activity are not yet completely elucidated.

Moreover, investigations on mercury effects have been mainly focused on the cardiovascular systems of normotensive animals. However, little information exists about the chronic effects of low doses of inorganic mercury regarding ACE activity in organs and tissues of normotensive and hypertensive animals. To investigate such effects, increased mercury levels were induced to produce blood level concentrations similar to those of exposed individuals. Therefore, we aimed to determine whether chronic exposure to inorganic mercury increases the activity of ACE and the relationship of such exposure with oxidative stress on heart, aorta, lung, brain and kidney in hypertensive compared to normotensive animals.

**Methods**

**Animals**

Three-month-old male normotensive Wistar rats and SHRs (spontaneously hypertensive rats) were obtained from the Federal University of Espirito Santo breeding laboratories. During treatment, rats were housed at a constant room temperature, humidity, and 12:12-h light-dark cycle. Rats had free access to tap water and were fed with standard chow ad libitum. All experiments were conducted in compliance with the guidelines for biomedical research as stated by Conselho Nacional de Controle de Experimentação Animal-CONCEA, and in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocols were approved by the Ethics Committee of Escola Superior de Ciências da Santa Casa de Misericórdia de Vitória, Brazil (CEUA-EMESCAM 003/2007). Wistar rats and SHRs were divided into four groups: control Wistar rats (n = 6) and SHRs (n = 9) treated with vehicle (saline solution, im), and Wistar rats (n = 8) and SHRs (n = 9) treated with mercury chloride (HgCl$_2$) for 30 days (1$^{\circ}$ dose 4.6 µg/kg, subsequent dose 0.07 µg/kg/day, im to cover daily loss). We used the model described by Wiggers et al.\textsuperscript{4} to reach blood level concentrations (7.97 ng/ml) similar to those of exposed individuals.

**Blood pressure measurements**

Indirect systolic blood pressure was measured at both the beginning and the end of the treatment using tail-cuff plethysmography (IITC Life Science Inc.). For this measurement, conscious rats were restrained for 5–10 min in a warm and quiet room and were conditioned to numerous cuff inflation-deflation cycles by a trained operator. Subsequently, systolic blood pressure was measured, and the mean of three measurements was recorded.

**Hemodynamic parameter measurements**

At the end of treatment, control and HgCl$_2$-treated rats (n = 26) were anaesthetized with urethane (1.2 g/kg, Sigma, St Louis, MO, USA), and the carotid artery and jugular vein were cannulated. A polyethylene catheter (PE50/Clay-Adams) filled with heparinized saline (50 U/ml) was introduced into the carotid artery to measure systolic blood pressure (SBP) and diastolic blood pressure (DBP). The carotid artery catheter was introduced into the left ventricle, and the jugular vein cannula was advanced into the right ventricular chamber to measure the left and right ventricular systolic pressures (LVSP and RVSP) and their positive and negative time derivatives (+dP/dt and -dP/dt, respectively) along with the left and right ventricular end-diastolic pressures (LVEDP and RVEDP). Recordings were performed over 30 min with a pressure transducer (TSD 104A-Biopac) and with an interface and software for computer data collection (MP100A, Biopac System, Inc., Santa Barbara, CA, USA). Heart rate (HR) was determined in the interbeat intervals.

Measurement of malondialdehyde (MDA) production. Levels of MDA in plasma, heart, aorta, brain, kidney and lung were measured using a modified thiobarbituric acid (TBA) assay.\textsuperscript{28} Plasma and tissue samples were mixed with 20% trichloroacetic acid in 0.6 M HCl (1:1, v/v), and tubes were kept on ice for 20 min to precipitate plasma components to avoid possible interferences. Samples were centrifuged at 1500 x g for 15 minutes before adding TBA (120 mM in Tris 260 mM, pH 7) to the supernatant in a proportion of 1:5 (v/v); then, the mixture was boiled at 97°C for 30 min. Spectrophotometric measurements at 535 nm were taken at 20°C. 

**ACE activity assay**

ACE activity was measured in plasma, heart, aorta, brain, kidney and lung using a fluorimetric method adapted from Friedland and Silverstein.\textsuperscript{29} Briefly, triplicate tissue and plasma samples (3 µL) were incubated for 15-90 minutes at 37°C with 40 µL of assay buffer containing the ACE substrate 5 mM Hip-His-Leu (Sigma). The reaction was stopped by the addition of 190 µL of 0.35 M HCl. The generated product, His-Leu, was measured fluorometrically following 10 min of incubation with 100 µL of 2% o-Phthalaldehyde in methanol. Fluorescence measurements were taken at 37°C in a FLUOstar Optima plate reader (BMG Labtech, Offenburg, Germany) with 350 nm excitation and 520 nm emission filters. The fluorescence plate reader was controlled using the FLUOstar Optima Software. Black 96-Well polystyrene microplates (Biogen Científica, Madrid, Spain) were used. A calibration curve with ACE from the rabbit lung (Sigma) was included in each plate.

**Data analysis and statistics**

The results are expressed as the mean ± SD. All parameters were tested for normality using the one-sample Kolmogorov-
Smirnov test. Differences were analysed using one-way ANOVA, followed by a post hoc Tukey test (GraphPad Prism Software, San Diego, CA). A p value < 0.05 was considered significant.

Results

At 30 days of mercury treatment, Wistar controls, Wistar treated rats, and treated and untreated SHRs had similar body weights, although the SHRs had lower body weights when compared with Wistar rats (Table 1).

Table 1 also shows that several organs, including the brain, heart, kidney and lungs, presented similar weights, normalized by body weight, which did not change after mercury treatment.

Indirect SBP measured at day zero in awake rats showed that SHRs had a higher mean arterial pressure compared with Wistar rats (Table 2). However, at the end of the treatment, mercury produced a significant increment of blood pressure only in HgCl2-treated SHR rats (Table 2).

Arterial blood pressures, ventricular pressures and their respective derivatives, and HR measurements in anaesthetized rats were not different between groups (Table 3), but the LVEDP increased after Hg treatment in the Wistar group, as previously reported. 17

It has been reported in animal and human studies that mercury increases free radical production leading to an oxidative stress.4,24,30,31 We then evaluated the oxidant state in the blood and in several other tissues by measuring MDA levels (Table 4). MDA plasma levels were greater in mercury-treated than in untreated Wistar rats but did not change in SHRs. Mercury treatment increased MDA levels in the heart in both Wistar and SHRs. In the aorta, different from plasma, MDA levels were increased in mercury-treated SHRs but not in Wistar rats. For brain and lungs, no changes were observed for MDA levels in mercury-treated Wistar rats, but a reduction occurred in SHRs. For kidneys, mercury treatment reduced MDA levels in both Wistar and SHR mercury-treated groups.

Since angiotensin II is reported to increase ROS and mercury increases ACE,13,32 we investigated whether ACE activity was altered after 30 days of mercury treatment in Wistar and SHR groups. Table 5 shows that plasma ACE activity was unaffected in both Wistar groups, although slightly reduced in HgCl2-treated SHRs might explain why the right ventricular pressures remained unchanged.

Table 1 – Body Weight (BW), Brain/BW, Heart/BW, Kidney/BW, Lung/BW, Adrenals/BW, Spleen/PC and Liver/PC from HgCl2-treated and non-treated Wistar rats and spontaneously hypertensive rats (SHRs)

|                          | Wistar Control n = 6 | Wistar HgCl2-treated n = 8 | SHR Control n = 9 | SHR HgCl2-treated n = 9 |
|--------------------------|----------------------|----------------------------|-------------------|------------------------|
| Body weight (BW) (g)      | 399 ± 58.3           | 384 ± 18.1                 | 216 ± 20.1**      | 222 ± 14.4**           |
| Brain/BW (mg/g)           | 4.58 ± 0.7           | 4.69 ± 0.5                 | 7.48 ± 0.7**      | 7.41 ± 0.5             |
| Heart/BW (mg/g)           | 3.06 ± 0.6           | 3.43 ± 0.2                 | 3.77 ± 0.2        | 3.81 ± 0.2             |
| Kidney/BW (mg/g)          | 6.44 ± 1.7           | 6.53 ± 0.5                 | 6.78 ± 0.3        | 6.80 ± 0.6             |
| Lung/BW (mg/g)            | 3.97 ± 1.5           | 4.53 ± 0.6                 | 6.48 ± 1.2**      | 7.91 ± 1.2**           |

Results represent mean ± SD; n: number of animals used. One-way ANOVA, post hoc Tukey’s. *p < 0.05 compared with the Wistar control and † p < 0.05 compared with HgCl2-treated Wistar rats.

Discussion

The results presented here suggest that Wistar rats and SHRs submitted to chronic exposure in inorganic mercury for 30 days, have blood concentrations similar to exposed individuals.1,2 In addition, HgCl2-treated SHR, but not Wistar rats have increased blood pressure at the end of treatment. The intervention also influenced ACE activity and oxidative stress, by increasing or decreasing them, mainly in SHRs.

Previous reports showed that changes resulting from chronic exposure to mercury have been focused on its toxic effects on the cardiovascular system and the associations with hypertension, carotid atherosclerosis, myocardial infarction and coronary heart disease.9,10,34 Mercury exposure, both acute and chronic, affects the heart and endothelial function, reducing NO bioavailability and increasing ACE and NADPH activities.15,18,19 Moreover, studies in rats showed that body weight gain and arterial pressure were not affected when chronic exposure was performed,1,17 suggesting that this treatment was not sufficient, in either amount or time, to produce changes. Our results reproduced those findings, showing no changes in body weight gain; additionally, similar behaviour was observed for the heart, brain, kidneys and lung, reinforcing the suggestion that this treatment is not sufficient to produce these changes, although cardiovascular function began to be affected.

Regarding the hemodynamic evaluation, no changes were observed in the left or right ventricle in Wistar rats or SHRs. Only an increment of LVEDP was observed in normotensive rats treated with mercury, indicating some deleterious effects of mercury on ventricular function.35 Right ventricular pressures were investigated because of our previous report showing that under acute mercury exposure (0.5 mg/kg), there was an increase in right ventricular systolic pressure because of pulmonary hypertension,1,36-39 which was not observed with chronic treatment in the present study. The fact that lung ACE activity was unaffected in both Wistar groups, although slightly reduced in HgCl2-treated SHRs might explain why the right ventricular pressures remained unchanged.
Table 2 – Values of systolic blood pressure (SBP in mmHg) measured by tail plethysmography in Wistar rats and spontaneously hypertensive rats (SHRs) before and after treatment for 30 days with HgCl₂.

|               | Wistar CT n = 5 | Wistar Hg n = 5 | SHR CT n = 5 | SHR Hg n = 5 |
|---------------|-----------------|-----------------|--------------|--------------|
| SBP – Day 0   | 123 ± 13        | 131 ± 15        | 205 ± 15     | 198 ± 22     |
| SBP – Day 7   | 119 ± 4         | 132 ± 9         | 221 ± 18     | 197 ± 18     |
| SBP – Day 14  | 115 ± 10        | 135 ± 9         | 219 ± 9      | 199 ± 29     |
| SBP – Day 21  | 132 ± 17        | 142 ± 14        | 200 ± 13     | 199 ± 9      |
| SBP – Day 30  | 117 ± 6         | 143 ± 11        | 220 ± 21     | 232 ± 19     |

Results represent the mean ± SD; n: number of animals used. One-way ANOVA, post hoc Tukey’s for all groups. *p < 0.05 vs. SHR treated with mercury at day 0

Table 3 – Hemodynamic parameters from untreated and mercury (HgCl₂)-treated Wistar rats and spontaneously hypertensive rats (SHRs)

|               | Wistar Control n = 6 | Wistar HgCl₂-treated n = 7 | SHR Control n = 6 | SHR HgCl₂-treated n = 7 |
|---------------|----------------------|-----------------------------|-------------------|-------------------------|
| SBP (mmHg)    | 105 ± 10             | 97 ± 11                     | 105 ± 7           | 113 ± 8                 |
| DBP (mmHg)    | 71 ± 10              | 67 ± 11                     | 58 ± 5            | 68 ± 11                 |
| HR (bpm)      | 324 ± 88             | 325 ± 58                    | 343 ± 32          | 341 ± 34                |
| LVSP (mmHg)   | 114 ± 20             | 107 ± 16                    | 117 ± 22          | 112 ± 8                 |
| LVEDP (mmHg)  | 0.256 ± 1           | 3.31 ± 1*                  | 1.11 ± 0.2        | 0.493 ± 0.5             |
| +dP/dt LV (mmHg/s) | 8627 ± 3378          | 8500 ± 2419                 | 7360 ± 1854       | 7001 ± 1921             |
| -dP/dt LV     | -6270 ± 1232        | -6249 ± 1234                | -7169 ± 1173      | -6524 ± 1131            |
| RVSP (mmHg)   | 32 ± 10              | 29 ± 5                      | 29 ± 5            | 33 ± 5                  |
| RVEDP (mmHg)  | -1,080 ± 1          | 1.10 ± 2                   | -0.472 ± 1       | 0.459 ± 0.3             |
| +dP/dt RV (mmHg/s) | 3339 ± 2202          | 1758 ± 435                  | 2776 ± 1056      | 2171 ± 405              |
| -dP/dt RV     | -2560 ± 1553        | -1,387 ± 469               | -1,833 ± 478     | -1,695 ± 368            |

Changes in systolic (SBP) and diastolic (DBP) pressure, heart rate (HR), left and right ventricle systolic pressure (LVSP, RVSP), left and right ventricle end diastolic pressure (LVEDP, RVEDP) and positive (+dP/dt) and negative first-time derivatives (-dP/dt) from the left and right ventricles of Control and HgCl₂-treated rats. The results represent the mean ± SD. n-Number of animals used. One-way ANOVA, post hoc Tukey’s. *p < 0.05 vs Wistar Control.

The reduction of NO bioavailability is a hallmark resulting from the increase in ROS generation contributing to the development of cardiovascular diseases such as atherosclerosis and hypertension.\(^{10,11,34}\) The interaction of superoxide anion with NO generates peroxynitrite that decreases NO bioavailability increasing vascular reactivity.\(^{20-22}\) In addition, it has been shown that an increase of the local ACE activity could increase NADPH oxidase activity\(^{16,40}\) and ROS in the aortas of normotensive and SHRs. Therefore, we investigated whether mercury effects alter the renin-angiotensin system and oxidative stress in the heart.

The increase in ACE activity induced by mercury could lead to increased activity of NADPH oxidase, which could, in turn, increase the release of ROS, generating an oxidative stress, as observed in this study.

Considering that both Hg and increased ACE activity can induce oxidative stress, we should observe a correlation between the amount of oxidative stress and ACE activity measured by MDA. An interesting aspect is that ACE activity levels and MDA concentrations showed similar behavior in plasma and organs investigated. Also, it is of note that both ACE activity and MDA concentrations showed more expressive changes in HgCl₂-treated SHRs. Similarly, inorganic mercury treatment aggravated hypertension in SHRs, suggesting that a pre-existing hypertensive condition enhances inorganic mercury action.

ROS are damped in the plasma of all locations where they are produced, and consequently, it is expected an increase in MDA. We have shown that plasma ACE activity increases after acute exposure to low mercury concentrations and reduces after exposure to high concentrations.\(^{16,29}\) However, we might speculate that in the SHR group, when exposed to mercury, tissues that produce more ROS, such as the aorta, lung and kidney, ACE activity is reduced. Similarly, in the brain tissue, which concentrates mercury, ACE activity also decreased. LVEDP increments in Wistar rats could be explained by the local increase in ACE activity and oxidative stress in the heart. These two factors might explain the small, but significant increase in LVEDP, probably induced by a calcium overload.

Although we cannot give a proper explanation for all the events, it can be suggested that mercury, even at concentrations that do not affect arterial pressure and weight gain in normotensive rats, affects ACE activity and oxidative stress. However, in hypertensive animals, inorganic mercury actions were more expressive. These findings give rise to questions that are not addressed by our results: can exposure to mercury inhibit ACE activity in situations where it is already increased? Does ACE activity in different organs...
Table 4 – Malondialdehyde (MDA) (mM/mg of protein) concentrations in plasma, heart, aorta, lung, brain and kidney of untreated and Mercury (HgCl₂)-treated Wistar rats and spontaneously hypertensive rats (SHRs)

|                | Wistar Control | Wistar HgCl₂-treated | SHR Control | SHR HgCl₂-treated |
|----------------|----------------|----------------------|-------------|------------------|
|                | n = 6          | n = 6                | n = 6       | n = 7            |
| Plasma         | 0.93 ± 0.15    | 1.28 ± 0.44*         | 0.89 ± 0.22 | 0.92 ± 0.05      |
| Heart          | 0.22 ± 0.03    | 0.28 ± 0.03*         | 0.45 ± 0.05 | 0.55 ± 0.05      |
| Aorta          | 0.13 ± 0.03    | 0.12 ± 0.05          | 0.96 ± 0.27 | 1.51 ± 0.37      |
| Lung           | 0.18 ± 0.05    | 0.14 ± 0.03          | 0.21 ± 0.03 | 0.12 ± 0.03      |
| Brain          | 0.13 ± 0.03    | 0.09 ± 0.03          | 0.54 ± 0.07 | 0.34 ± 0.03      |
| Kidney         | 0.38 ± 0.07    | 0.14 ± 0.03*         | 0.96 ± 0.07 | 0.51 ± 0.03      |

Values are expressed in mM/mg of protein (MDA). The results represent the mean ± SD. N-Number of animals used. One-way ANOVA, post hoc Tukey’s. *p < 0.05 vs Wistar Control and &p < 0.05 vs SHR Control.

Table 5 – Angiotensin converting enzyme (ACE) activity levels in plasma, heart, aorta, lung, brain and kidney of untreated and Mercury (HgCl₂)-treated Wistar rats and spontaneously hypertensive rats (SHRs)

|                | Wistar Control | Wistar HgCl₂-treated | SHR Control | SHR HgCl₂-treated |
|----------------|----------------|----------------------|-------------|------------------|
|                | n = 6          | n = 6                | n = 6       | n = 6            |
| Plasma         | 187 ± 39.2     | 235 ± 34.3*          | 114 ± 27.9* | 163 ± 38.7*      |
| Heart          | 3.4 ± 0.5      | 4.1 ± 0.3*           | 17.9 ± 2.7* | 14.8 ± 1.4*      |
| Aorta          | 213 ± 53.9     | 221 ± 61.3           | 670 ± 39.9* | 535 ± 47.0*      |
| Lung           | 95 ± 6.1       | 99.4 ± 11.3          | 87.6 ± 5.4  | 75.1 ± 9.8*      |
| Brain          | 46.4 ± 7.9     | 42.6 ± 9.9           | 40.3 ± 5.6  | 27.8 ± 4.4*      |
| Kidney         | 47.8 ± 16.2    | 45.4 ± 14.2          | 80.0 ± 15.4*| 61.4 ± 6.9*      |

Values are expressed in nmol/mL/min/mg of protein in tissues and in nmol/mL of plasma/min in plasma (ACE). Results represent the mean ± SD. N-Number of animals used. One-way ANOVA, post hoc Tukey’s. *p < 0.05 vs Wistar Control and &p < 0.05 vs SHR Control.

Conclusions

Results described here allow us to affirm that chronic exposure to inorganic mercury, similarly to that we previously reported, produces blood concentrations compatible with those found in exposed humans, and do represent a cardiovascular risk factor. Such exposure influenced ACE activity, increased oxidative stress and promoted hypertension in SHRs (which had a higher blood pressure increment compared with untreated SHRs), as well as increased the LVEDP in Wistar rats. This controlled exposure affected the cardiovascular system, produced more expressive changes of ACE activity and oxidative stress in SHRs representing a risk factor for the development of cardiovascular disorders in normotensive rats and a contributing factor to pre-existing risks in high blood pressure condition.

Author contributions

Conception and design of the research: Vassallo DV, Simões MR, Giuberti K, Stefanon I; acquisition of data: Giuberti K, Azevedo BF, Ribeiro Junior RF; analysis and interpretation of the data and statistical analysis: Vassallo DV, Simões MR, Giuberti K, Azevedo BF, Ribeiro Junior RF, Salaices M, Stefanon I; obtaining funding: Vassallo DV, Salaices M; writing of the manuscript: Vassallo DV, Simões MR; critical revision of the manuscript for intellectual content: Vassallo DV, Simões MR, Salaices M, Stefanon I.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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Ethics approval and consent to participate

This study was approved by the Ethics Committee on Animal Experiments of the Escola Superior de Ciências da Santa Casa de Misericórdia de Vitória under the protocol number CEUA-EMESCAM 003/2007.
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