Cardiovascular harmful effects of recommended daily doses (13 µg/kg/day), tolerable upper intake doses (0.14 mg/kg/day) and twice the tolerable doses (0.28 mg/kg/day) of copper

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Abstract

Copper is essential for homeostasis and regulation of body functions, but in excess, it is a cardiovascular risk factor since it increases oxidative stress. The objective of this study was to evaluate the effects of exposure to the recommended daily dose (13 μg/kg/day), upper tolerable dose (0.14 mg/kg/day) and twice the upper tolerable dose (0.28 mg/kg/day) via i.p. over 4 weeks on the vascular reactivity of aortic rings and the contraction of LV papillary muscles. It was also determined whether the antioxidant peptide from egg white hydrolysate (EWH) prevents these effects. Copper exposure at the doses evaluated did not change weight gain, the reactivity of the aortic rings or the cardiac mass. The dose of 0.13 μg/kg/day did not reduce the force of contraction, but it impaired the time derivatives of force. Doses of 0.14 and 0.28 mg/kg/day reduced the force of contraction, the inotropic response to calcium and isoproterenol, the postrest contraction and the peak and plateau of tetanized contractions. EWH treatment antagonized these effects. These results suggest that copper, even at the dose described as upper tolerable, can impair cardiac contraction without altering vascular reactivity. Antioxidative stress therapy with EWH reversed these harmful effects, suggesting a possible strategy for the amelioration of these effects.

Introduction

Copper (Cu) is a mineral present in our daily lives and is an essential factor for human homeostasis. It is widely used in industries due to its ability to act as a conductor of electricity and heat, used as a civil construction material and as a component of various metal alloys [1]. It is considered essential for the physical and mental health of humans, and it is a vital cofactor in several enzymes, such as cytochrome C oxidase, lysyl oxidase and superoxide dismutase. In addition, it acts as an electron donor in redox reactions [2, 3]. However, copper overload can be toxic to living beings [4–6].

Copper toxicity occurs when there is excessive dietary supplementation, consumption of contaminated food and water, exposure to copper intrauterine devices, agricultural spraying, or occupational exposure in mining or metallurgical industries. Occupational exposure to copper occurs during work with fungicides, paints, alloys, construction materials and the copper smelting and handling industries [1]. However, previous studies have shown that the safe and toxic limits of copper can be quite close. The US Institute of Medicine and the WHO recommend a daily intake of 0.9 mg/day of copper for adults with a body weight of 70 kg (13 μg/kg/day of copper) and mention a safe upper limit [7, 8].

Freire et al. [9] reported that hospitalized adults showed that increasing copper intake from 0.83 to 0.96 mg/day was directly related to an increased incidence of ischemic heart failure. In addition, another study in humans showed that an increase in blood copper concentration from 0.72 to 0.97 μg/mL, due to environmental exposure, increased the occurrence of myocardial infarction seven-fold [11, 12]. Other studies have also shown a relationship between slight increases in the serum copper concentration and the occurrence of heart failure and death from cardiovascular diseases [13–15].

Most of the toxic effects of copper are related to increased oxidative stress by reactive oxygen species (ROS) causing oxidative damage [16]. Excess free radicals in the body are neutralized by antioxidant agents produced by the body, as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase stand out. In addition to endogenous antioxidants, several studies point to the beneficial effects of consuming various natural antioxidant products and bioactive food ingredients, such as protein-derived peptides, to protect against oxidative imbalance [17–20]. In this functional food group, the bioactive peptides from egg white hydrolysate (EWH) have demonstrated important antioxidant properties, preventing dysfunction in experimental animal models exposed to metals such as mercury and aluminum [18–20].

Although the recommended doses of copper are well established, the limits between safe and toxic doses of this metal are still not well established. Therefore, we aimed to evaluate the effects of the recommended daily dose (13 µg/kg/day), the maximum tolerable intake dose (0.14 mg/kg/day) and twice the tolerable dose (0.28 mg/kg/day) of copper for 4 weeks on the cardiovascular system of rats. In addition, we evaluated whether the administration of egg white hydrolyzed peptide (EWH) can provide protection against the harmful effects of copper.

Materials And Methods

Animals

The use of animals was carried out in accordance with the guidelines of the National Council for the Control of Animal Experiments [21]. Our protocols were approved by the animal ethics committee (CEUA 26/2018).

Wistar rats (*Rattus norvegicus*) of approximately 280 grams and 12 weeks of age were used. They were kept in standard cages on a 12-hour light-dark cycle with temperature control and free access to water and food.

Experimental Model

To create the experimental model, three copper concentrations were chosen based on the recommendations of the Institute of Medicine [7] and the World Health Organization [8], which recommend a daily intake of 0.9 mg of copper for adults of 70 kg, equivalent to 13 µg/kg/day. A daily dose of 10 mg of copper for humans, or 0.14 mg/kg/day, is considered the maximum tolerable copper intake level [22]. While this dose can have adverse gastrointestinal effects [23], there are no reports of it causing cardiovascular damage. Therefore, we also evaluated whether double the maximum tolerated dose (0.28 mg/kg/day) can have adverse effects on the cardiovascular system.

Thus, the animals were randomly divided into four groups: a control group (Ct, N = 6) and three groups of 6 animals per group exposed to different copper concentrations (13 µg, 0.14 mg, or 0.28 mg). Therefore,

animals in the control group received 0.1 mL of saline intraperitoneally (i.p.). for 4 weeks, while animals in the 13 µg, 0.14 mg and 0.28 mg/kg/day copper groups received copper chloride i.p. for 4 weeks.

Weight assessment

The weights of the animals in the different experimental groups were measured before the start of the experiment and weekly thereafter to assess whether exposure to different levels of copper could influence their body weight gain over 4 weeks.

The weight parameters of the cardiac chambers were obtained at the end of the 4 weeks of copper exposure. To obtain these data, the right and left ventricles were dissected, washed in saline solution (NaCl 0.9%), dried on paper towels to remove the excess liquid and weighed. To avoid possible “false results” at the expense of the size of the ventricles, the weight of the chambers was corrected by the length of the tibia of each corresponding animal as described by Fioresi et al. [24].

Vascular reactivity

At the end of the exposure period, animals from different groups were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) i.p. The thoracic aortas were dissected, and all fat and connective tissue was removed. Then, the aortas were cut into rings approximately 4 mm long, and the preparations were placed in organ baths containing Krebs-Heinseleit solution (in mM: NaCl 127, KCl 4.7, $CaCl_2$ 2H2O 2.5, $MgSO_4$ 7H2O 1.2, KH_2PO_4 1.17, $NaHCO_3$ 24, glucose 11, 0.01 EDTA, pH 7.4), aerated with a carbogenic mixture (5% CO_2 and 95% O_2), and maintained at 37°C. The isometric tension was recorded with an isometric force transducer (TSD125C, BIOPAC Systems, Goleta, CA, USA) connected to a data acquisition system (MP100, BIOPAC Systems). The aortic rings remained in a resting tension of 1 g for 45 min of stabilization and were readjusted when necessary. Then, the aortic rings were evaluated twice with 75 mM KCl; the first exposure was made to verify its functional integrity, and the second exposure measured the maximum developed force. Then, the contractile response to phenylephrine (Phe, 10 µM) was evaluated.

Evaluation of the contractility of isolated papillary muscles

After anesthesia, the hearts were removed and perfused with a Krebs-Henseleit solution containing (in mM): 135 NaCl, 4.6 KCl, 1.25 $CaCl_2$, 1.15 $MgSO_4$, 1.2 KH_2PO_4, 5.5 glucose, pH = 7.4. The papillary muscles of the posterior wall of the left ventricle were carefully dissected, and the ends were fixed in stainless steel rings, placed in a bath containing 20 mL of Krebs-Henseleit solution, constantly aerated with carbogenic solution (95% oxygen and 5% carbon dioxide), and then kept at 29°C as described by Vassallo et al. [25].

The preparations were connected to an isometric force transducer (TSD125 - Biopac Systems, Inc. CA). Electric field stimulation was induced by isolated pulses (10 to 15 V, duration 5 ms) that were applied through a pair of platinum electrodes placed along the entire length of the muscle. The standard rate of stimulation was 0.5 Hz [25].

Then, the papillary muscle was successively stretched until reaching the maximum contraction force, called *Lmax*. The muscle was kept in this condition, performing stable contractions for 60 minutes, to stabilize it under the new experimental conditions.

Evaluations of isometric contractions

To assess the possible effects induced by exposure to the recommended daily doses, tolerable upper intake doses and twice the tolerable doses of copper on the contractility of papillary muscles, the force of contraction, the time to peak tension, the time to 90% relaxation and the positive and negative time derivatives of force (+ dF/dt and -dF/dt) were measured in all experimental groups.

The value of the contraction force was measured through the relationship between the contraction amplitude in grams and the muscle weight in milligrams. Time to peak tension and 90% relaxation times were measured as the time spent from the onset of contraction to the maximum peak force and the maximum peak to 90% of the isometric relaxation.

Indirect assessment of sarcoplasmic reticulum activity

This protocol was performed to indirectly assess the possible effects of exposure to 13 µg, 0.14 mg and 0.28 mg/kg/day of copper on the activity of the sarcoplasmic reticulum. Relative postpause potentiations (PPPs) were obtained by pausing the electrical stimulation at intervals of 15, 30 and 60 seconds [25]. The PPPs were calculated through the relationship between the force of the first contraction after electrical restimulation and the stabilized contraction force before the pause.

Evaluation of the inotropic response to extracellular calcium

To assess the possible changes induced by 13 µg, 0.14 mg and 0.28 mg/kg/day copper in response to extracellular calcium, the force of contraction of the papillary muscles was measured at concentrations of 0.62, 1.25 and 2.5 mM calcium chloride. The inotropic response was evaluated by the maximum amplitude of the stabilized contraction after increasing the extracellular calcium concentration.

Indirect evaluation of sarcolemmal calcium influx

This protocol was performed to assess whether exposure to 13 µg, 0.14 mg or 0.28 mg/kg/day of copper could alter the sarcolemmal calcium influx. For this, a calcium-free Krebs-Henseleit solution containing caffeine (5 mM) was used. At this concentration, caffeine keeps the ryanodine channel open [26, 27], depleting the calcium stores present in the sarcoplasmic reticulum (SR) [25]. Then, the electrical stimulation was interrupted for 10 minutes. Two minutes before electrical restimulation, the Krebs-Henseleit nutrient solution containing 1.25 mM $CaCl_2$ was returned. Thus, postrest contraction (PRC) depended exclusively on sarcolemmal calcium influx. The PRC was calculated from the amplitude of the first contraction after electrical restimulation corrected by the stabilized contraction force [28].

Assessment of the inotropic response to beta-adrenergic agonists

To investigate whether exposure to 13 µg, 0.14 mg or 0.28 mg/kg/day copper could interfere with the inotropic response to a beta-adrenergic receptor agonist, incubation with isoproterenol at a concentration of 10^{-4} M in the bath was performed. The inotropic response was evaluated by the maximum amplitude of the stabilized contraction after incubation with isoproterenol.

Evaluation of the tetanized contractions

The protocol of tetanic contractions was carried out to evaluate the effects of exposure to 13 µg, 0.14 mg or 0.28 mg/kg/day of copper on the contractility of papillary muscles with dysfunctional SR [27]. For this, Krebs-Henseleit solution containing calcium chloride (1.25 mM) and caffeine (5 mM) was used for 30 minutes; thus, muscle contraction became dependent on the sarcolemmal calcium influx and on the sensitivity of contractile proteins to this ion [26, 27]. At the end of this period, the frequency of electrical stimulation was increased to 10 Hz for 15 seconds [27]. Thus, a tetanized contraction was generated that started with a peak of contractile force, followed by the contraction plateau. For experimental purposes, we measured the force developed at the peaks and plateaus of the tetanic contractions.

Evaluation of the antioxidant role of hydrolyzed egg white peptides (EWH)

The EWH was obtained through pepsin hydrolysis of egg white, as described by Garcés-Rimón et al. [29]. Briefly, pasteurized egg white was hydrolyzed with BC Pepsin 1:3000 (EC 3.4.23.1; from pig stomach, E:S: 2:100 p:p, pH 2.0, 38°C), purchased from Biocatalysts (Cardiff, UK), for 8 hours. Enzyme inactivation was achieved by raising the pH to 7.0 with 5 N NaOH. The hydrolysate was centrifuged at 2500 x g for 15 min, and the supernatants were frozen and lyophilized.

Copper and EWH treatment

After exposure to copper at the three doses described above, new groups of male Wistar rats were acquired, and the effects of simultaneous copper and EWH administration were evaluated. The antioxidant role of EWH was tested in the group of animals that received an equivalent to a daily dose of 10 mg of copper for humans (0.14 mg/kg/day), as our previous results show that this dosage reduces the force of contraction of the papillary muscles. Rats were divided into four groups and received the following treatments for 4 weeks: the Ct group received 0.9% saline i.p. and tap water by gavage; the Cu group received 0.14 mg/kg/day of copper chloride i.p. and tap water by gavage; the EWH group received 0.9% saline i.p. and EWH diluted in tap water at a dose of 1 g/kg/day by gavage, based on previous work [30]. The Cu and EWH groups received both treatments, copper by intramuscular injection and EWH by gavage.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). One-way ANOVA followed by Fisher's post test was used for comparisons between groups. Two-way ANOVA followed by Bonferroni post test was used to analyze the vascular reactivity, postpause potentiation and the response to

extracellular calcium. Values of $P < 0.05$ were considered significant. Data analysis and figure construction were performed using GraphPad Prism 8.0 System (San Diego, CA, USA).

Results

Effects of exposure to different copper doses

Animals in the control group and in those that received the recommended daily dose, tolerable upper intake dose and twice the tolerable dose of copper started and ended the treatment with similar weights, suggesting that copper exposure in the proposed experimental model was not able to change body weight gain (Fig. 1).

Vascular reactivity induced by phenylephrine

To evaluate the possible effects of 4 weeks of exposure to 13 µg, 0.14 mg and 0.28 mg/kg/day of copper on vascular reactivity, a concentration–response curve to phenylephrine was obtained. The contractions of the aortic rings were similar among the different experimental groups evaluated, suggesting that exposure to copper in this experimental model is not capable of altering vascular reactivity (Fig. 2).

Effects of copper exposure on RV, LV, and papillary muscle mass

At the end of the experimental exposure period, the RV, LV and papillary muscles were dissected, and their masses were measured and corrected for the length of the tibia. There were no differences among the groups in terms of RV, LV and papillary muscle weights, suggesting that the treatments did not promote myocardial hypertrophy or cardiac remodeling (Fig. 3A, B and C).

Effects of copper exposure on papillary muscle contractility

To assess whether exposure to different concentrations of copper could impair cardiac excitation-contraction coupling, the contractile parameters were evaluated. Our results showed that exposure to copper led to a reduction in the contraction force of the papillary muscles. Figure 4A shows the reduction in cardiac contractile force after treatment for 4 weeks with 0.14 mg and 0.28 mg/kg/day copper.

The times to peak tension (TTP) and to 90% relaxation (TR 90%) allow us to infer the kinetics of the mechanisms that mobilize and remove calcium from the sarcoplasm during processes involving excitation-contraction coupling in the LV papillary muscles. Figure 4 shows that TTP did not change for any of the copper treatments performed, whereas TR 90% was prolonged after 4 weeks of exposure to 0.28 mg/kg/day of copper (Fig. 4B and C).

The positive and negative time derivatives of the force (dF/dt) were also measured. Positive and negative dF/dt were depressed after exposure to the three doses of copper evaluated (Fig. 4D and E). This result

shows that although the force of contraction decreases only at doses of 0.14 mg/kg/day and 0.28 mg/kg/day, dF/dt also decreases with 13 µg/kg/day treatment, suggesting that the harmful effect on the contractile performance produced by copper is already visible at the recommended daily doses.

Evaluation of the regulatory mechanisms of cardiac contractility

To assess the mechanisms involved in reducing the contraction induced by exposure to 13 µg, 0.14 mg and 0.28 mg/kg/day of copper, an indirect assessment of SR activity was performed. It was observed that none of the copper treatments were able to change the SR function, since the relative potentiation of papillary muscles from the groups exposed to copper remained similar to the control group after pauses of 15, 30 and 60 seconds (Fig. 5A).

To investigate the effects of copper exposure on the inotropic responses of papillary muscles, we constructed a concentration–response curve of the changes in extracellular calcium concentration. Figure 5B shows that there was a reduction in the contractile response of the papillary muscles from groups exposed to 13 µg, 0.14 mg and 0.28 mg/kg/day of copper chloride when compared to the control group.

To assess whether the reduction in the inotropic response found in groups exposed to copper could be related to changes in calcium influx through sarcolemmal calcium channels, PRC was performed. According to our results, the PRC of the papillary group of animals exposed to 0.14 mg/kg/day of copper was reduced compared to controls. However, in the other groups exposed to copper, the response was similar to that in the control group (Fig. 5C).

Figure 5D shows the effects of inotropic intervention caused by the β-adrenergic receptor agonist isoproterenol. A positive inotropic response was present but was reduced in the papillary muscles of animals exposed to 13 µg, 0.14 mg and 0.28 mg/kg/day copper for four weeks.

Figure 5E and F shows the reduction in peak tetanic force in groups exposed to the three doses of copper compared to controls, while the plateau of tetanic contractions was impaired in groups of animals exposed for 4 weeks to 13 µg and 0.14 mg/kg/day of copper, while the group that received 0.28 mg/kg/day was similar to the control group.

According to our previous data (see Fig. 4A), exposure to tolerable upper intake doses of copper (0.14 mg/kg/day) induced a reduction in the contraction force of papillary muscles, and these effects may be at least in part related to increased oxidative stress. Therefore, we used an antioxidant, EWH, to ameliorate oxidative stress in this experimental group. The results showed that cotreatment with EWH was able to restore the contraction force that had been impaired in papillary muscles from rats exposed to copper (Fig. 6).

Discussion

This study stress the possible occurrence of noxious effects caused by exposure to the recommended daily dose, tolerable upper intake dose and twice the tolerable upper intake level of copper on cardiac contractility. Copper exposure did not change weight gain, vascular effects leading to pressure overload, or promoted cardiac hypertrophy in the animals at any dose evaluated. The amount equivalent to 10 mg of copper/day for humans of 70 kg, although considered safe by regulatory agencies, reduced the cardiac force generation, the time derivatives of force, the response to extracellular calcium, the β-adrenergic response, the postrest contraction and tetanic contractions. Similar effects were obtained after exposure to an amount equivalent to 20 mg/day of copper in adults, which is considered twice the tolerable upper intake levels for this metal. The amount equivalent to 0.9 mg/day of copper, despite causing changes in some of the regulatory mechanisms of cardiac contractility, was not able to reduce the force generation of papillary muscles.

It was observed that the body weight gain during the 4 weeks of exposure was similar among the experimental groups that received 13 μg, 0.14 mg or 0.28 mg/kg/day of copper, corroborating a study by Naseri et al. [31] and Mattiolli et al. [32], in which steers that received copper supplementation up to the maximum recommended level showed no change in body mass gain.

To investigate whether the cardiac effects were not due to other factors, such as vascular effects leading to pressure overload, we tested the copper effects on aortic segments. As the aorta is a vessel that readily reflects the effects of metals producing oxidative stress [33–35], the fact that these three doses did not alter vascular reactivity reinforces the idea that the effects found in this study are restricted to the heart only. Therefore, we did not proceed with any vascular studies because no changes were found.

Cardiac hypertrophy is one of the most serious risk factor for cardiovascular disease. In our findings, exposure to or above the recommended levels of copper was not able to change the weight of the right and left ventricles or the papillary muscles of rats. We know that copper acts as a cofactor of cytochrome c oxidase and lysyl oxidase, and increased exposure to copper improves the activity of these enzymes [36]. Zheng et al. [36] reported that increased cytochrome c oxidase activity prevents cardiac hypertrophy. Rodrigues and González [37] reported that an increase in copper concentration improves the function of lysyl oxidase, maintaining myocardial extracellular matrix homeostasis through the oxidation of collagen and elastin chains and preventing the occurrence of cardiac hypertrophy.

The evaluation of force, time to peak tension, time to 90% relaxation, and positive and negative time derivatives of force suggest that exposure to the tolerable upper intake levels and twice the tolerable upper intake levels of copper were able to reduce inotropism and affect the temporal parameters of contraction. According to our data, these effects seem to be related to reduced extracellular calcium influx through the sarcolemma and reduced activity of cardiac contractile proteins [38]. We know that increased exposure to copper is capable of impairing the contractility of papillary muscles and their regulatory mechanisms due to the increased formation of reactive oxygen species (ROS) [39]. Other studies have described the occurrence of changes in cardiac function in situations of increased exposure and/or

elevated blood concentration of copper, but they do not explain the underlying mechanisms responsible for these effects [40–43].

Copper leads to the formation of free radicals from the chemical reactions of Fenton and Haber-Weiss [44]. Previous reports have shown that oxidative stress development is the main mechanism by which copper impairs cardiac contractility [45–51]. Based on this, we performed a detailed assessment of each of the regulatory mechanisms of cardiac contractility to describe a model of cardiotoxicity induced by exposure to increasing levels of copper. We used the recommended daily doses, tolerable upper intake levels, and twice the tolerable upper intake levels.

Calcium influx assessed through postrest contraction decreased after exposure to 0.14 mg/kg/day copper, while the inotropic response to extracellular calcium decreased in groups of animals exposed to all doses evaluated. These results suggest that copper reduces calcium influx through L-type calcium channels. Previous studies evaluating exposure to toxic metals such as iron and lead showed that ROS impairs the mechanisms that make calcium available for myocardial contractility, including calcium influx through sarcolemmal L-type channels [52, 53]

Another analysis of our study was to assess whether exposure to copper doses could influence the response to a β-adrenergic agonist. The β-adrenergic agonist promotes a series of second messenger-mediated effects on cardiomyocytes that culminate in phosphorylation of phospholamban (PLBp) and L-type calcium channels, resulting in greater permeability to extracellular calcium and increasing calcium reuptake by SERCA, resulting in increased force of contraction and accelerated relaxation. This response was significantly diminished in papillary muscles from animals exposed to the recommended daily doses, tolerable upper intake levels, and twice the tolerable upper intake levels. Kaneko et al. [54] investigated the effects of ROS on α- and β-adrenergic receptors in rat hearts, which suggested that free radicals are able to modify β-adrenergic receptors and downregulate them, causing a decrease in the inotropic response.

Tetanic contractions were used to assess the cardiac contractile machinery with the dysfunctional sarcoplasmic reticulum; thus, the contraction becomes dependent on the influx of calcium and the sensitivity of contractile proteins [24, 53]. The developed peak and plateau forces of the tetanic contractions of the LV papillary muscles were impaired by exposure to doses of 13 μg, 0.14 mg and 0.28 mg/kg/day of copper. A previous study by Filetti et al. [39] also showed a reduction in tetanic contraction after acute exposure to high concentrations of copper, which in this case was related, at least in part, to a reduction in myosin ATPase activity. In fact, Moreira et al. [55] evaluated the effects of other toxic metals and showed the ability of divalent metals to bind to the SH radicals of the myosin molecule, reducing ATP hydrolysis and consequently reducing contraction. Furthermore, other studies have demonstrated the ability of copper to bind to SH radicals in other body tissues [56, 57].

It has already been shown that oxidative stress is the main mechanism by which copper impairs cardiac contractility [45–51] thus, the effects of antioxidant treatment with EWH during copper exposure were evaluated. Although a dose of 13 μg/kg/day of copper altered some regulatory mechanisms of cardiac

contraction, the force of contraction remained stable, while in the groups exposed to doses of 0.14 and 0.28 mg/kg/day of copper, the force of contraction was reduced. In addition, a dose of 0.14 mg/kg/day of copper is considered safe and easy to obtain in exposed populations, so the evaluation of the antioxidant role of EWH was performed only in the group that received the equivalent of 10 mg/day of copper in humans.

The presence of tyrosine and phenylalanine in EWH is related to the neutralization of free radicals [58]. In addition, previous studies have shown that EWH is able to reduce peroxidation, one of the mechanisms that causes lipid damage, in addition to increasing SOD expression and reducing NADPH oxidase expression [18–20, 59]. Finally, EWH can be considered an ingredient in functional foods and can be used as an additional therapy in the treatment of cardiovascular toxicity promoted by copper.

Conclusion

In conclusion, our study stress for the first time the possible occurrence of harmful cardiac effects of copper caused by oxidative stress, effects that were not due to vascular effects leading to pressure overload. Results compared the effects of the recommended daily dose (13 µg/kg/day), tolerable upper intake dose (0.14 mg/kg/day) and twice tolerable upper intake dose (0. 28 mg/kg/day). The findings showed that copper exposure does not alter vascular reactivity and that the toxic effects are restricted to the heart. Despite the copper dose of 13 µg/kg/day altering some of the mechanisms that regulate contraction, there was no reduction in the contractile force of the papillary muscles. The doses of 0.14 mg and 0.28 mg/kg/day of copper reduced the force of contraction and impaired the mechanisms that regulate contraction, and these effects may be related to the increased generation of oxygen species. We also showed that although 0.14 mg/kg/day of copper is considered a safe dose by regulatory agencies, there is a need for review, considering the harmful effects that this dose was able to produce. These findings indicate that EWH may represent an effective public health strategy, as it can be used as a functional or complementary therapy in the treatment of damage caused by copper exposure.

Declarations

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

FUNDING

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Author contributions

Filipe Martinuzo Filetti: wrote and developed most of the project, performed the treatment of the animals with copper and hydrolyzed egg white, assessed the contractility of the papillary muscles, analyzed and interpreted the results, prepared figures 1, 3, 4, 5 and 6, and wrote the article.

Ingridy Reinholz Grafites Schereider: Collaborator on the project, she performed the assessment of vascular reactivity and contributed to the analysis and interpretation of the results, and prepared figure 2.

Giulia Alessandra Wiggers: Collaborated with the interpretation of results and discussion of results.

Marta Miguel: Collaborated with the interpretation of results and discussion of results.

Dalton Valentim Vassallo: Co-author of the research project, he guided the development of the entire study, participated in the analysis of the results, reviewed all the figures and contributed to the writing of the article.

Maylla Ronacher Simões: Was responsible for the project, participated as an advisor in all phases of experimentation, in the analysis of results, in the guidance of the following stages, reviewed all figures and was also the writer of the article.

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Figures

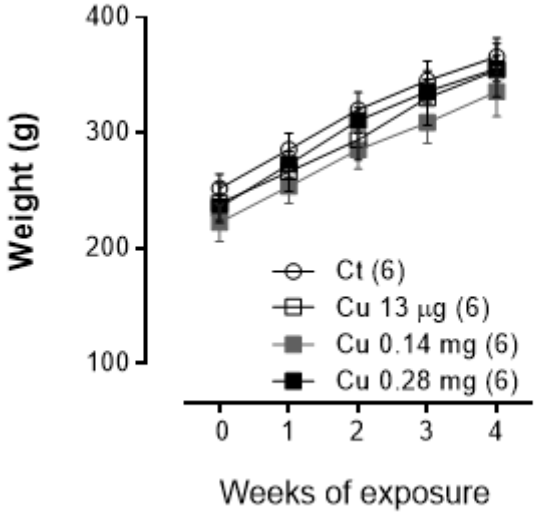

Figure 1

Weight evaluation after 4 weeks of exposure to 13 µg, 0.14 mg and 0.28 mg/kg/day copper. The numbers in parentheses represent the number of samples evaluated. Values represent the mean ± SEM. Two-way ANOVA was used

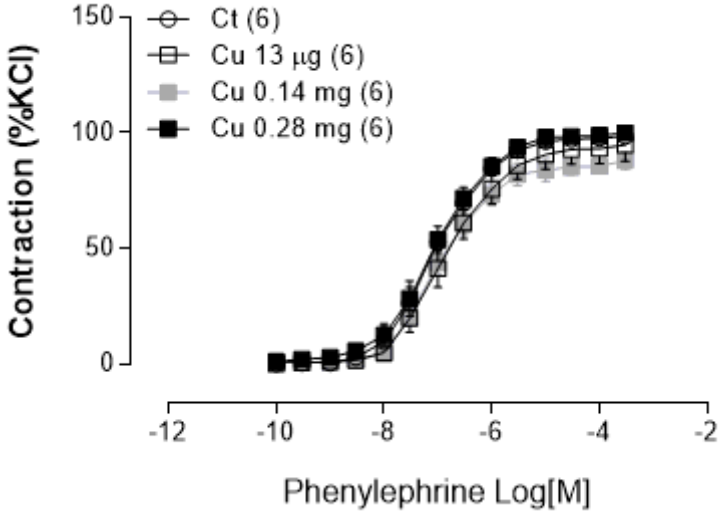

Figure 2

Vascular reactivity induced by phenylephrine in the aortic rings was not altered after 4 weeks of exposure to 13 µg, 0.14 mg or 0.28 mg/kg/day of copper. The numbers in parentheses represent the number of samples evaluated. Values represent the mean ± SEM. Two-way ANOVA was used

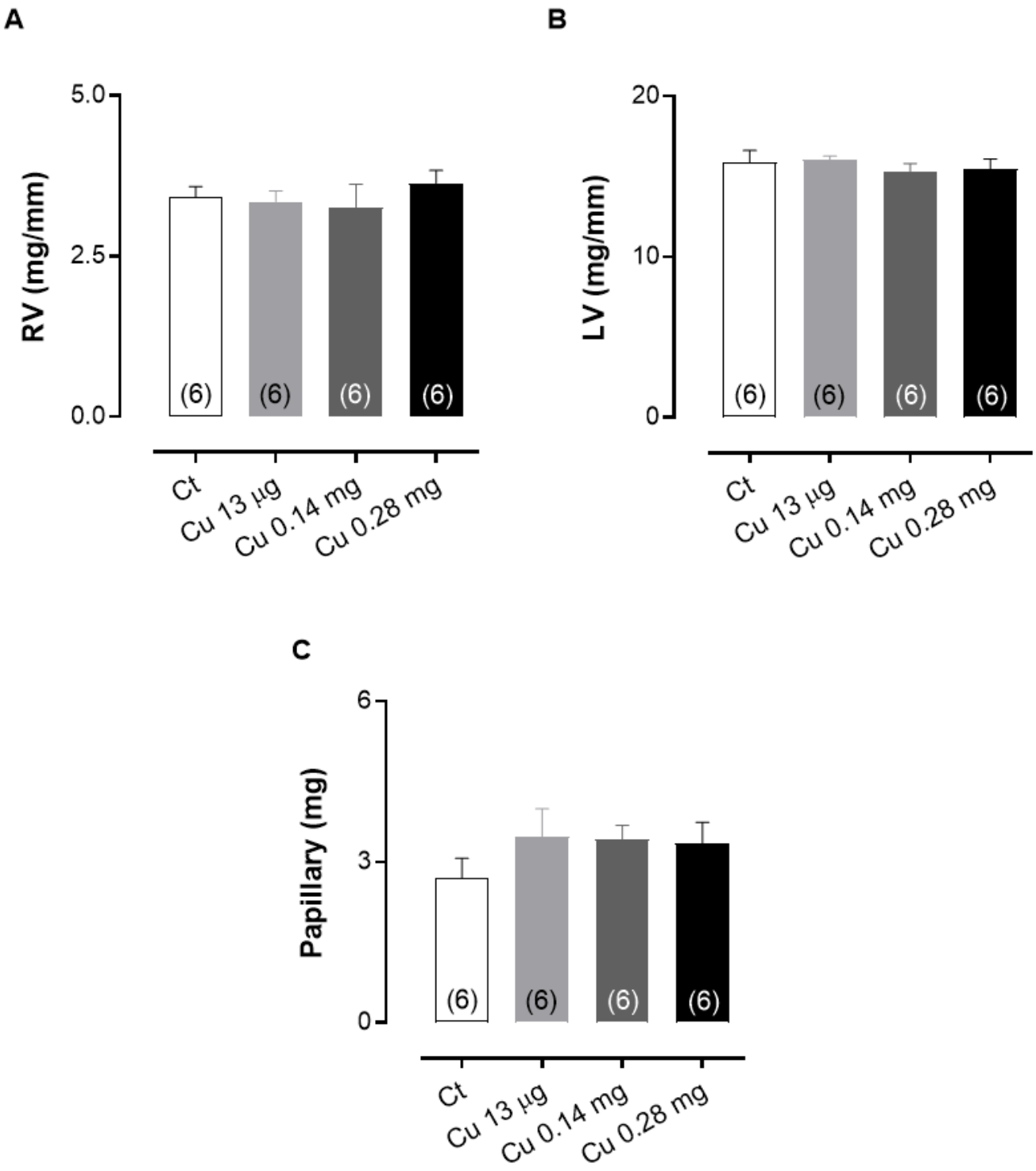

Figure 3

Weight evaluation after 4 weeks of exposure to 13 µg, 0.14 mg and 0.28 mg/kg/day of copper. A: Right ventricular weight corrected for tibia length. B: Left ventricular weight corrected for tibia length. C: Weight of the papillary muscles. The numbers in parentheses represent the number of samples. Values represent the mean ± SEM. One-way ANOVA was used

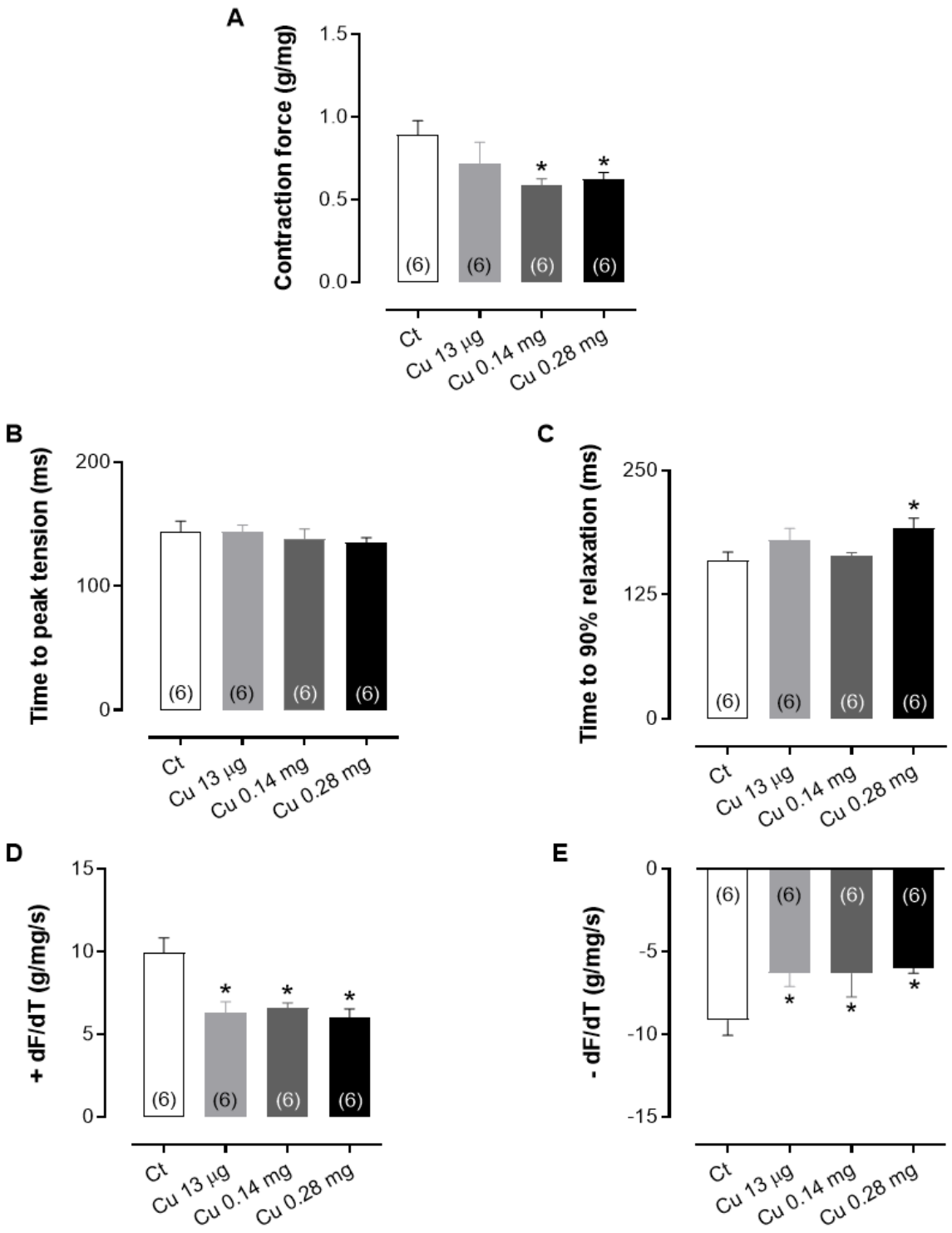

Figure 4

Baseline parameters of papillary muscle contraction after 4 weeks of exposure to 13 µg, 0.14 mg and 0.28 mg/kg/day of copper. A: contraction force. B: Time to peak tension. C: Time for 90% relaxation. D. Time derivative of the maximum positive force. E: Time derivative of the maximum negative force. The

numbers in parentheses represent the number of samples. *P < 0.05 vs. Ct. Data are expressed as the mean ± SEM. One-way ANOVA followed by Fisher's post-test

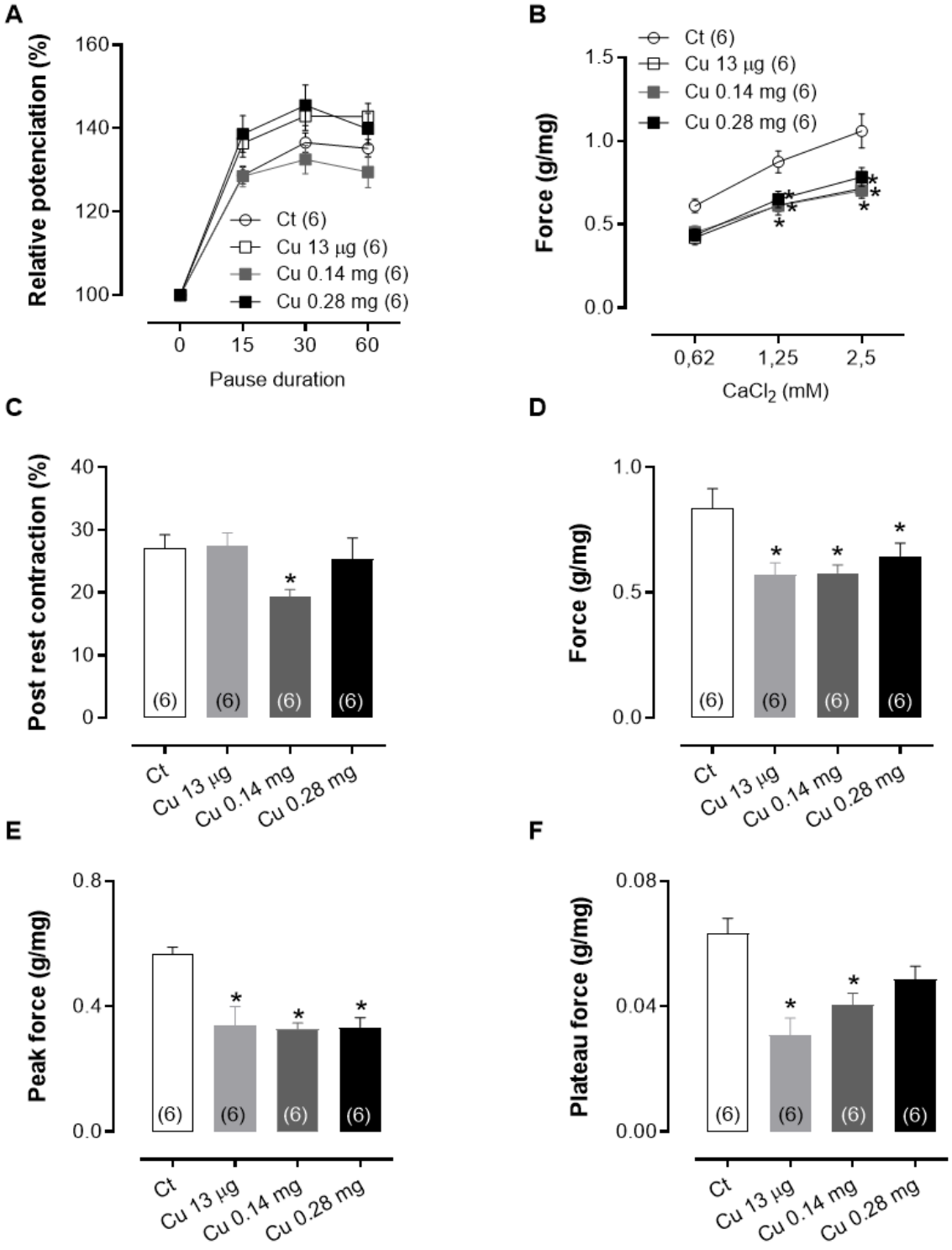

Figure 5

Inotropic interventions in the papillary muscles after 4 weeks of exposure to 13 µg, 0.14 mg or 0.28 mg/kg/day of copper. A: Relative potentiation after pauses. B: Inotropic intervention induced by different calcium concentrations. C: Postrest contractions obtained after 10 min without stimulation in a calcium-free solution containing 5 mM caffeine. D: Inotropic intervention induced by isoproterenol. E: Relative tetanic peak force, and F: Relative tetanic plateau force. The numbers in parentheses represent the number of samples. Data are expressed as the mean ± SEM. *P < 0.05 vs. Ct. Two-way ANOVA followed by Bonferroni post test in A and B. One-way ANOVA followed by Fisher's post test from C to F

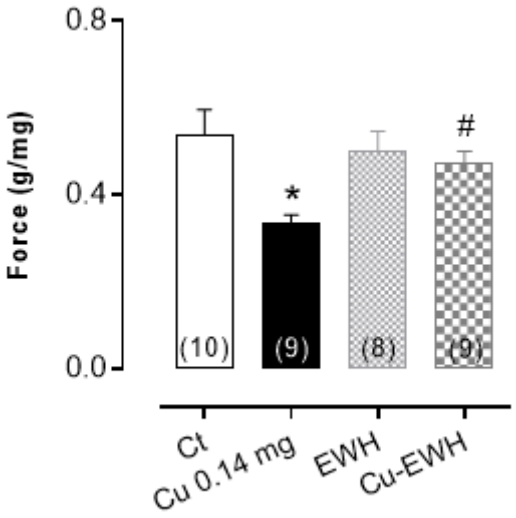

Figure 6

Effects of 4 weeks of exposure to copper (0.14 mg/kg/day) and/or hydrolyzed egg white peptides (EWH) on rat LV papillary muscle strength development. The numbers in parentheses represent the number of samples. Values represent the mean ± SEM. *P < 0.05 vs. Ct, #P < 0.05 vs. Cu. 1-way ANOVA followed by Fisher's post test