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Copper exposure at twice the recommended daily dose prevents impairment in cardiac contraction strength in diabetic rats despite elevated free radical levels

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Abstract

Individuals with diabetes often present a higher risk of cardiovascular diseases and imbalances in Cu metabolism. Moreover, copper supplementation is widely used by the general population. The combined effects of copper (Cu) exposure and diabetes (DM) on cardiac function are not yet fully understood. This study investigated the impact of chronic exposure to $CuCl_2$, at a dose twice the recommended daily intake, on the cardiac contractile function of diabetic and non-diabetic rats. Male Wistar rats were divided into four groups: control (Ct), copper (Cu), diabetic (DM), and copper-diabetic (Cu-DM). Type 1 diabetes was induced by a single dose of streptozotocin (65 mg/kg, i.p.), and animals treated with Cu received 25.7 µg/kg/day of $CuCl_2$ for 30 days. We assessed cardiac function by measuring left ventricular papillary muscle contractions. Both copper treatment and diabetes reduced contraction strength, altered the cardiac calcium handling, and increased free radical production. However, although contraction strength was preserved, this effect was accompanied by oxidative stress. Therefore, although copper prevented the negative inotropic effect induced by diabetes, its use as a therapeutic strategy should be approached with caution, as the functional benefit is accompanied by increased free radical generation.

Introduction

Diabetes mellitus (DM) has become a growing epidemic in recent decades and is now one of the leading causes of death worldwide [1]. In 2019, diabetes was the direct cause of 1.5 million deaths, with nearly half (48%) of these deaths occurring prematurely, before the age of 70 [2]. DM is a significant risk factor for the development of cardiovascular diseases, which are the leading cause of death among individuals with DM; in people with heart failure and reduced ejection fraction, DM is further associated with a greater burden of hospitalizations due to decompensated heart failure, other cardiovascular events, and infections, with infections making the largest contribution [1, 3]. Cardiovascular complications are frequently associated with oxidative stress, which may also be triggered by other contributing factors [4].

Among the risk factors that can influence the development of oxidative stress, as well as the onset and progression of diabetes, are disturbances in copper (Cu) homeostasis [5]. Cu is an essential metal for the body, necessary for the functioning of antioxidant enzymes and normally obtained through a healthy diet [6]. However, Cu exposure can occur through industrial activity, in the form of environmental residues, through insecticides [7], and often in vitamin supplements, widely used by individuals with diabetes [8].

Excessive Cu exposure can lead to an imbalance in the body's blood concentrations of this metal, becoming a risk factor for the development of oxidative stress and the progression of cardiovascular diseases and diabetes [9, 10]. Experimental studies have shown that exposure to both tolerable and high doses of Cu affect cardiac contractility by altering calcium homeostasis and promoting oxidative stress [11, 12, 13]. It is known that both diabetes and excessive Cu supplementation cause oxidative stress [14] which is related to impairments in myocardial contractility [12].

Given the interplay between Cu metabolism, oxidative stress, and diabetes-related cardiovascular complications, it is essential to investigate how Cu supplementation affects cardiac function in diabetic conditions. Despite the widespread use of Cu-containing supplements and the potential for excess exposure in the general population, the impact of combined Cu exposure and diabetes on cardiac function remains poorly understood.

Therefore, this study aimed to investigate the impact of chronic exposure to $CuCl_2$, at a dose twice the recommended daily intake, on the cardiac contractile function of diabetic and non-diabetic rats.

Materials and methods

Animals

The care and use of the animals adhered to the guidelines for biomedical research established by the Brazilian Societies of Experimental Biology. The experimental procedures were approved by the local Research Ethics Committee (CEUA/UFES 22/2019).

Wistar rats (*Rattus norvegicus albinus*), weighing about 280 g and aged 12 weeks, were housed in cages, maintained on a 12-hour light-dark cycle, with temperature control, and had free access to food and water. These animals were randomly assigned to four experimental groups: control group (Ct), copper-exposed group (Cu), diabetic group (DM), and diabetic copper-exposed group (Cu-DM). The rats in the copper-exposed group received daily intraperitoneal (i.p.) injections of $CuCl_2$ at a dose of 25.7 μg/kg/day for 30 days, corresponding to approximately twice the recommended daily intake when adjusted for body weight [10, 15, 16]. Each group was housed separately to prevent the control animals from being exposed to water and waste from the Cu-treated animals. This protocol is considered a chronic or subchronic exposure and is commonly used in studies of Cu and other metal toxicities [16, 17]. The intraperitoneal route was chosen to ensure precise control of the administered dose and to minimize variability associated with gastrointestinal absorption and first-pass metabolism, allowing more consistent systemic exposure.

The selected dose was based on a translational approach derived from the recommended daily intake for humans corresponding to approximately twice the recommended intake when adjusted for body weight. This experimental model has been previously validated by our group in diabetic rats [10, 15].

Experimental model of diabetes

Experimental diabetes was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ) at a dose of 65 mg/kg body weight. STZ, a drug that induces diabetes, was dissolved in normal saline solution 12 hours prior to administration. To ensure the rats became diabetic, fasting blood sugar concentration was measured after 48 hours. Male Wistar rats with blood sugar levels exceeding 200

mg/dl were considered diabetic. After the confirmation of diabetes, treatment with Cu chloride was initiated, lasting 30 days [18, 10].

Body weight

The animals were weighed prior to the start of treatment, weekly, and at the conclusion of the 30-day Cu exposure period to determine if Cu overload would impact the weight gain in both diabetic and non-diabetic rats.

Evaluation of contractility of isolated papillary muscles

To assess the impact of Cu exposure on myocardial contractility in both diabetic and non-diabetic rats, we measured the isometric force of isolated papillary muscles. Anesthetized animals underwent a thoracotomy, and their hearts were excised and perfused with Krebs-Henseleit solution (composition in mM: NaCl 120; KCl 5.4; $CaCl_2$ 1.25; $MgCl_2$ 1.2; NaH_2PO^4 2; Na_2SO^4 1.2; NaHCO3 24; and glucose), which was oxygenated with 5% O_2 and 95% CO_2 to maintain a pH of 7.4. The left ventricular papillary muscles were dissected and mounted in glass chambers with a volume of 20 mL, secured between a fixed point and a force transducer. The experiments were conducted at 26 ºC to minimize the risk of hypoxia [19].

The papillary muscles were electrically stimulated using platinum electrodes placed along their length, delivering 12 ms rectangular pulses at 10 to 15 V. The standard stimulation frequency was set at 0.5 Hz. The muscles were then stretched to the length at which the active tension was maximal (Lmax). The preparations were allowed to stabilize for at least 40 minutes. Contractile force was recorded with an isometric force transducer (TSD125 - Biopac Systems, Inc.; CA) connected to an amplifier (DA100C Biopac Systems, Inc.; CA) and a data acquisition system (MP100 Biopac Systems, Inc.; CA). Following the stabilization period, the experimental protocol commenced.

Isometric contraction and contractile kinetics

To examine whether Cu exposure affects the contractile activity of the papillary muscles in diabetic and non-diabetic rats, we measured the following parameters in both groups: force development (F), activation and relaxation times, and positive (+ dF/dt) and negative (-dF/dt) force derivatives.

The developed force was normalized by the ratio of contraction amplitude in grams (g) to muscle weight in milligrams (mg) to account for variations due to differences in muscle size. Activation and relaxation times were recorded as the duration from the beginning of contraction to the peak force and from the peak force to 90% of isometric relaxation, respectively [20].

Indirect evaluation of sarcoplasmic reticulum activity

The post-pause potentiation (PPP) protocol was conducted to indirectly evaluate the effects of 30-day Cu exposure on the functional activity of the sarcoplasmic reticulum (SR) in both diabetic and non-diabetic rats. This involved applying electrical stimulation to the muscle after pauses of 15, 30, and 60 seconds. PPP was assessed as relative potentiation, calculated as the ratio of the contraction amplitude after the pause to the contraction amplitude before the pause. This procedure was used to normalize changes in pre-pause contractions across different muscle preparations [12, 21].

Contractile effects by changes in extracellular calcium concentration

To determine if Cu exposure modified the contractile response to calcium in diabetic and non-diabetic rats, we examined the response to various calcium concentrations (0.62, 1.25, and 2.5 mM). The inotropic response was assessed by measuring the maximum amplitude of the stabilized contraction following the increase in extracellular calcium concentration [12, 21].

Indirect measurement of sarcolemmal calcium influx by “post rest contraction-PRC”

The purpose of this protocol was to evaluate how Cu exposure affects sarcolemmal calcium influx in both diabetic and non-diabetic rats. Post-rest contractions (PRC) were measured after a 10-minute period without stimulation. The muscles were immersed in a modified calcium-free Krebs solution containing caffeine (5 mM). At this concentration, caffeine depletes the SR's calcium stores by keeping the ryanodine channels open [21, 22]. Since sarcolemmal calcium pumps remove myoplasmic calcium during the non-stimulation period, the first contraction after the rest period relies solely on calcium influx through the sarcolemma [22]. Two minutes prior to resuming electrical stimulation, the solution was replaced with Krebs-Henseleit containing 1.25 mM Ca^{2+}. The contractions after the rest period were calculated as a percentage of the first post-pause contraction relative to the amplitude of the steady-state contraction before the pause.

Evaluation of contractile response to β-adrenergic stimulation

To determine if Cu exposure affected the cardiac response to β-adrenergic receptors, we incubated the muscles with isoproterenol, a non-selective β-adrenergic agonist, at a concentration of 10 − 4 M in the bath. Following stabilization of the preparation, the inotropic response was evaluated by measuring the maximum amplitude of the stabilized contractions after isoproterenol incubation.

Evaluation of contractile response in tetanic contractions

Tetanic contractions of papillary muscles were induced by exposing them to Krebs-Henseleit solution containing 5 mM caffeine for 30 minutes. This procedure ensures the contractile response occurs

without a functional SR [21, 22, 12]. As a result, the contractile activity relies solely on sarcolemmal Ca^{2+} influx and the responsiveness of contractile proteins to Ca^{2+}. We investigated the effects of Cu exposure on tetanic contractions of papillary muscles in both diabetic and non-diabetic rats, using a stimulation rate of 10 Hz for 15 seconds. For experimental analysis, we measured the force developed at the peaks and plateaus of the tetanic contractions.

In situ evaluation of the production of O2•-, NO and H_2O_2

To explore the role of superoxide anion (O2•-), nitric oxide (NO), and hydrogen peroxide (H_2O_2) production in the harmful effects induced by Cu exposure in diabetic and non-diabetic animals, we utilized fluorescence techniques involving the oxidation of dihydroethidium (DHE), diaminofluorescein (DAF), and 2',7'dichlorofluorescein diacetate (DCF).

Tissue samples from frozen left ventricular papillary muscle segments were sliced into 10-µm-thick sections and placed on glass slides. Successive sections were treated under identical conditions for 30 minutes at 37°C in Krebs-HEPES buffer (comprising 130 mM NaCl, 5.6 mM KCl, 2 mM CaCl2, 0.24 mM MgCl2, 8.3 mM HEPES, and 11 mM glucose, pH 7.4). Fresh buffer containing DHE (2 M) and HPF (10 µM) was applied to each tissue section, covered with a coverslip, and incubated for 30 minutes in a light-protected humidified chamber at 37°C. Subsequently, the sections were observed using a fluorescent laser-scanning confocal microscope (Leica TCS SP2 equipped with a krypton/argon laser, x40 objective) with consistent imaging settings for both control and treated groups. Images from both groups were captured using a Leica DM 2500 fluorescence microscope equipped with a Leica DFC 310 FX camera. Five frozen tissue segments per animal were analyzed for each experimental condition, and the mean fluorescence densities in the targeted region were quantified using ImageJ 1.44p software.

Western blot analysis

Heart proteins from homogenized samples were separated via 10% SDS-PAGE and then transferred to nitrocellulose membranes. Subsequently, they were incubated overnight with monoclonal antibodies as follows:

- SERCA-2a: anti-SERCA2 ATPase mouse monoclonal antibodies at a dilution of 1:1000 (ThermoScientific, Rockford, USA).
- Phospholamban (PLB): mouse monoclonal anti-PLB antibody at a dilution of 1:1000 (Santa Cruz, California, USA).
- Phospho-Ser16-phospholamban (phospho-Ser16-PLB): polyclonal rabbit anti-phospho-Ser16-PLB antibody at a dilution of 1:1000 (Badrilla, West Yorkshire, UK).

Following washing steps, the membranes were treated with anti-rabbit or anti-mouse immunoglobulin antibody conjugated to anti-mouse, anti-rabbit, or anti-goat IgG immunoglobulin (diluted 1:5000, Sigma Aldrich), and peroxidase-conjugated Streptactin (diluted 1:5000, Bio-rad, California, USA). Protein bands were visualized through a chemiluminescence reaction using a detection system (ECL Plus, Amersham

TM, GE Healthcare, UK) and captured by a ChemiDoc XRS+ transilluminator (Bio-rad, California, USA) with optimized exposure settings. Densitometric analysis was performed to quantify protein bands using Image Lab 6.1 software (Bio-rad, California). The densitometry results of each protein band of interest were normalized by quantifying the total protein content of each sample obtained through UV light stimulation.

Statistical analysis

The results were expressed as mean ± standard error of the mean. The "n" values indicate the number of animals used in each experimental protocol. Statistical analysis was conducted using unpaired Student's t-test and/or one or two-way analysis of variance (ANOVA). If ANOVA yielded significant results, Fisher's post-test was employed to compare means. Values of $P < 0.05$ were deemed statistically significant. Data analysis and figure plotting were carried out using the GraphPad Prism System (San Diego, CA, USA) and GB-STAT (Dynamic Microsystem Inc., Silver Spring, MD, USA).

Results

Validation of the diabetic model

Blood glucose levels confirmed the successful induction of diabetes, with diabetic groups (DM and Cu-DM) showing significantly higher values compared to control groups both at baseline (post-induction) and at the end of the experimental period (Table 1).

Table 1
Blood glucose levels before and after experimental period.

Group	Blood glucose (mg/dL) at baseline	Blood glucose (mg/dL) at the end of the experimental period
Ct	125 ± 3.4	172 ± 13.1
Cu	119 ± 5.3	177 ± 19.4
DM	421 ± 35 $^{\& \#}$	482 ± 21.5 $^{\& \#}$
Cu-DM	447 ± 32.2 $^{\& \#}$	558 ± 18.1 $^{@ \& \# *}$

Values are expressed as mean ± SEM. One-way ANOVA was used for single-parameter comparisons, and two-way ANOVA for initial vs. final blood glucose. Tukey post-hoc and Fisher's LSD tests were applied where appropriate ($p < 0.05$). & vs Ct; # vs Cu; * vs DM; @ intragroup difference. Initial measurements were performed after diabetes induction (n = 10).

Myocardial contractility

The wet weight of the left ventricle (LV) papillary muscles was obtained at the end of the experimental protocols. The weights of the papillary muscles were not altered in any experimental group (results not

shown). In animals subjected to the combination of diabetes and Cu exposure, myocardial contraction strength was similar to that of the control group (Ct) (Fig. 1a). However, in the Cu and DM groups, the developed force was lower compared to the Ct group. Regarding the temporal parameters of contraction and relaxation of the papillary muscle exposed to Cu, there was an increase in activation time in the diabetic group (Cu-DM) compared to the Ct, Cu, and DM groups, while the relaxation time of muscle contraction was shorter in the Cu and DM groups. However, the Cu-DM group maintained a relaxation time similar to the Ct group (Figs. 1b and 1c). The indices of contractility and relaxation, positive and negative dF/dt (Figs. 1d and 1e), did not show significant changes in all experimental groups, except for the Cu group, which showed a decrease in negative dF/dt compared to the other groups.

Indirect assessment of sarcoplasmic reticulum activity

For the indirect evaluation of sarcoplasmic reticulum (SR) activity, the post-rest potentiation (PRP) protocol with pauses of 15, 30, and 60 seconds was used [25]. In animals subjected to the combination of diabetes and Cu exposure, PRPs were similar to those of the control animals (Ct) (Fig. 2a). However, in the Cu and DM groups, the developed PRPs were lower compared to the other groups, suggesting that the combination of Cu and DM promotes increased calcium reuptake/release from the SR.

Evaluation of the inotropic response to extracellular calcium

Another intervention carried out to investigate the effects of the combination of diabetes and Cu exposure on the regulatory mechanisms of myocardial contractility was a concentration-response curve to changes in extracellular Ca^{2+} concentration. Figure 2b indicated that alterations in extracellular calcium did not affect the inotropic response of the papillary muscles of the different experimental groups, suggesting that despite the greater availability of calcium in the DM + Cu group, as shown in Fig. 2a, the contractile proteins seem to respond less to this availability.

Indirect assessment of sarcolemmal Ca^{2+} influx

This protocol allowed the evaluation of the permeability of the sarcoplasmic membrane to Ca^{2+}. Long-term exposure to zero calcium in the nutrient solution, plus caffeine (5 mM), which empties the SR calcium stores, activates the sarcolemmal calcium pumps during this period without stimulation to deplete the myoplasmic calcium. Thus, if we replace the normal solution with calcium, the first contraction after the rest will depend exclusively on the influx of sarcolemmal calcium. In animals subjected to the combination of diabetes and Cu exposure, PRC was similar to that of the control animals (Ct) (Fig. 3a). However, in the Cu and DM groups, PRC was lower compared to the other groups, suggesting that animals subjected to the combination of Cu and DM have the calcium influx through the sarcolemmal membrane restored to values equal to the Ct group.

Evaluation of contractile response to β-adrenergic stimulation

In order to evaluate whether the combination of diabetes and Cu promotes alterations in sympathetic modulation and consequently alters the regulatory mechanisms of myocardial contractility, the papillary muscles were subjected to acute exposure to a β-agonist, isoproterenol. Upon exposure to isoproterenol, the DM and Cu-DM groups showed responses similar to the Ct group (Fig. 3b), indicating an equal inotropic response to sympathetic stimulation. These findings suggest that this pathway is not related to the restoration of strength and the increased release of Ca^{2+} by the SR, nor does it seem to be associated with the increased influx of Ca^{2+} through the sarcoplasm.

Evaluation of tetanic contractions

To investigate the sarcolemmal Ca^{2+} influx and contractile protein sensitivity, a protocol of tetanic contractions was performed. Figures 3c and d show that changes in maximum tetanic force were not altered among the different experimental groups; however, the plateau of tetanic contractions was reduced in the DM and Cu-DM groups, suggesting that the reduction in protein sensitivity present in diabetes persisted in the Cu-DM association. Nonetheless, this reduction did not impair cardiomyocyte force, as shown in Fig. 1a. Moreover, since there appears to be an increased supply of Ca^{2+} in the sarcoplasm (Figs. 2a and 2c), this may cause a compensatory effect to this reduction, resulting in the maintenance of myocyte force (Fig. 1a).

In situ detection of free radicals and nitric oxide

To investigate whether the association of diabetes with Cu alters free radical formation, fluorescence in situ assessment was conducted to determine superoxide anion (O2●-), nitric oxide (NO-), and hydrogen peroxide (H_2O_2) production by incubating with DHE, DAF, and DCF, respectively. Superoxide anion, nitric oxide, and hydrogen peroxide production were found to be increased in the Cu, DM, and DM + Cu groups (Fig. 4a, 4b, and 4c). However, DCF fluorescence demonstrated that the combination of diabetes with Cu increased H_2O_2 (Fig. 4c).

Biochemical Evaluation

Biochemical evaluation was performed to assess the impact of Cu exposure on proteins involved in calcium handling, including SERCA2, PLB, and pPLB, which regulate cardiac contractility. Western blot analysis of left ventricular papillary muscle proteins (Fig. 5) suggested that animals subjected to the Cu and DM combination showed restoration in the expression of proteins involved in calcium handling in cardiomyocytes, as the DM condition promoted increased PLB expression and reduced pPLB expression. This mechanism supports the maintenance of contractile force observed in the DM + Cu group (Fig. 1a).

Discussion

This study explored the impact of Cu overload on the cardiac function of diabetic rats. The rats were exposed to twice the recommended daily Cu dose for 30 days, and diabetes was induced using streptozotocin. Both Cu treatment and diabetes reduced the contraction strength of left ventricular papillary muscles, altered the cardiac calcium handling, and increased free radical production. The combined exposure to copper and diabetes prevented the negative inotropic effect observed in isolated cardiac muscle compared to the Cu and DM groups evaluated individually. This effect was accompanied by increased H_2O_2. Our findings suggest that, under diabetic conditions, copper supplementation stimulates calcium-dependent mechanisms of myocardial contraction, particularly those related to calcium homeostasis, and may compensate for diabetes-induced dysfunction, thereby preserving myocardial contractility in isolated preparations. However, this effect was accompanied by oxidative stress.

The copper dose used in this study (25.7 µg/kg/day) was selected based on a translational approach derived from the recommended daily intake for humans, corresponding to approximately twice the recommended intake when adjusted for body weight. This strategy is commonly employed to model conditions of moderate excess exposure. Furthermore, this dosing regimen has been previously validated by our research group in diabetic rats, demonstrating significant metabolic and vascular effects, supporting its biological relevance in the present experimental context [10, 15].

Previous research has demonstrated that exposure to the recommended daily dose of Cu in non-diabetic rats did not reduce contraction strength but impaired the time derivatives of force. In contrast, exposure to the tolerable upper intake level and twice the tolerable upper intake level of Cu reduced contraction strength, inotropic response to calcium and isoproterenol, post-rest contraction, and the peak and plateau of tetanic contractions [23].

One of the main features of diabetic cardiomyopathy is diastolic dysfunction, affecting the cardiac muscle's relaxation capacity. This is linked to alterations in the calcium sensitivity of contractile proteins and changes in myosin isoforms [24]. Troponin phosphorylation in diabetic cardiomyopathy also plays a role in reducing cardiac muscle contractility. Moreover, insulin metabolic signaling is impaired, reducing glucose uptake, increasing calcium concentration in cardiac cells, and affecting nitric oxide production, essential for regulating cardiac function [25, 26].

The protocols that investigated transsarcolemmal calcium influx and calcium reuptake/release from the SR suggested that the cardiomyocytes isolated from the animals in the Cu-DM group were more permeable to calcium than those isolated from the Cu and DM group and displayed enhanced calcium cycling activity, with values restored to those of the control group. These mechanisms may explain the avoided decreased of contraction strength to levels similar to those in the control group.

Although transsarcolemmal calcium influx was elevated in the Cu-DM group, contractile force during tetanic stimulation did not increase, suggesting persistent impairments in calcium sensitivity. However,

the decreased sensitivity of proteins associated with diabetes persisted in the Cu-DM group. Nevertheless, this reduction in the sensitivity of the contractile proteins did not impair cardiomyocyte strength. While sympathetic modulation may influence cardiac performance via increased calcium mobilization or altered protein sensitivity, it does not appear to explain the inotropic responses observed in the Cu-DM group.

Biochemical evaluation assessed the impact of Cu exposure on calcium-handling proteins, including SERCA2, PLB, and pPLB, which regulate cardiac contractility. Western blot analysis of left ventricular papillary muscle indicated that animals in the Cu-DM group showed restored expression of these proteins, with the DM condition increasing PLB and decreasing pPLB expression. This restoration supports the maintenance of contractile force observed in the Cu-DM group.

Copper exposure improved cardiac contractile parameters in diabetic animals while increasing oxidative stress markers. These findings should be interpreted as parallel rather than causally linked effects. Diabetes-induced cardiac dysfunction involves alterations in calcium handling [25, 26], and the improvement observed here may be associated with changes in calcium-handling proteins identified in our model. Previous studies have shown that copper can influence cardiac contractility and excitation–contraction coupling [12, 13]. In contrast, the increase in oxidative stress is consistent with the redox properties of copper [6]. Together, these findings support the context-dependent effects of copper under diabetic conditions [5].

Both Cu exposure and diabetes have been associated with an increased incidence of cardiovascular diseases [27, 28]. Diabetic cardiomyopathy, characterized by changes in the structure and function of the cardiac muscle, represents a notable complication of diabetes.[24] Overexposure to Cu can disrupt the metal's concentration in the blood, making it a risk factor for oxidative stress and contributing to the advancement of cardiovascular diseases and diabetes [29].

In this study, the findings related to the increased production of superoxide anion, nitric oxide, and hydrogen peroxide in the Cu, DM, and Cu-DM groups may be closely related to the reduced sensitivity of contractile proteins in papillary muscle fibers to calcium. This phenomenon can be attributed to an increase in oxidative stress, which is commonly observed in conditions such as diabetes mellitus [30] and the presence of excess Cu [12].

The concurrent increase in superoxide anion, nitric oxide, and hydrogen peroxide likely exacerbates calcium dysregulation, further impairing muscle protein contractility in diabetic and Cu-exposed hearts [31]. This suggests that strategies targeting oxidative stress may enhance calcium sensitivity and cardiac performance in patients with diabetes and altered Cu metabolism.

The DCF fluorescence showed that the combination of diabetes and Cu increased hydrogen peroxide levels compared to the other groups. In this context, studies have demonstrated that hydrogen peroxide (H_2O_2) can contribute to the improvement of cardiac contraction strength by modulating cellular

signaling pathways that influence calcium handling and the function of contractile proteins in the heart [32, 33].

At sublethal concentrations, H_2O_2 can act as a signaling molecule that modifies the phosphorylation of key proteins involved in cardiac contractility, such as phospholamban (PLB) and SERCA2, which can result in increased calcium release from the sarcoplasmic reticulum and, consequently, enhanced contractile force [33].

According to a meta-analysis, the supplementation of Cu appears to offer potential benefits in the treatment of type 2 diabetes mellitus. Thirteen controlled randomized clinical trials (RCTs) were reviewed, assessing the effects of Cu supplementation in people with type 2 diabetes (DM2). Cu supplementation was associated with a significant reduction in fasting blood glucose levels (FBG), hemoglobin A1c (HbA1c), and improved insulin sensitivity [34]. Moreover, it was demonstrated that Cu plays a vital role in glucose regulation and protection against oxidative damage. Additionally, Cu is essential for the normal function of pancreatic beta cells and glycogen synthesis [9]. These factors may be related to the improvement of cardiac function in diabetics supplemented with Cu.

This study has some limitations that should be acknowledged. First, the use of the intraperitoneal route of copper administration does not fully replicate the most common route of human exposure, which is oral, and therefore may influence the translational interpretation of the findings. Second, although the selected copper dose was based on a translational approach and previously validated by our group, no pilot study was conducted specifically for this experimental design. Third, glycated hemoglobin (HbA1c), an important marker of long-term glycemic control, was not evaluated, as the study focused on short-term experimental outcomes. Finally, the mechanisms underlying the dissociation between improved cardiac contractility and increased oxidative stress were not fully elucidated and warrant further investigation. Despite these limitations, the study provides relevant insights into the complex effects of copper exposure under diabetic conditions.

In conclusion, chronic Cu exposure and diabetes independently impaired cardiac contractility and calcium handling, and increased free radical production. However, their combination paradoxically preserved myocardial contractile function, likely through restored expression of calcium-handling proteins. Despite this functional benefit, free radical levels were exacerbated. These findings highlight the dual role of copper in cardiac physiology, suggesting therapeutic potential under strict control. Further research is needed to validate these results and define safe, effective supplementation protocols for individuals with diabetes. As a limitation, this study was conducted only in male animals; however, new studies from our group are being developed in females to clarify potential sex-related differences.

Declarations

Ethical approval

The care and use of the animals adhered to the guidelines for biomedical research established by the Brazilian Societies of Experimental Biology. The experimental procedures were approved by the local Research Ethics Committee (CEUA/UFES 22/2019).

Data Availability

The datasets generated during and/or analyzed during the current study are included within the manuscript.

Credit authorship contribution statement

Mirian Fioresi: Data curation, formal analysis, investigation, methodology, project administration, writing - original draft, and writing - review & editing.

Julia Antonietta Dantas: Data curation, formal analysis, investigation, methodology, project administration, writing - original draft.

Karoline Neumann - Data curation, formal analysis, investigation, methodology, project administration, writing - original draft.

Filipe Martinuzo Filetti: Conceptualization, data curation, formal analysis, investigation, methodology, project administration roles/writing - original draft and writing - review & editing.

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Karolini Zuqui Nunes: Conceptualization, data curation, formal analysis, funding acquisition, project administration, resources, supervision, validation, visualization, roles/writing - original draft, writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

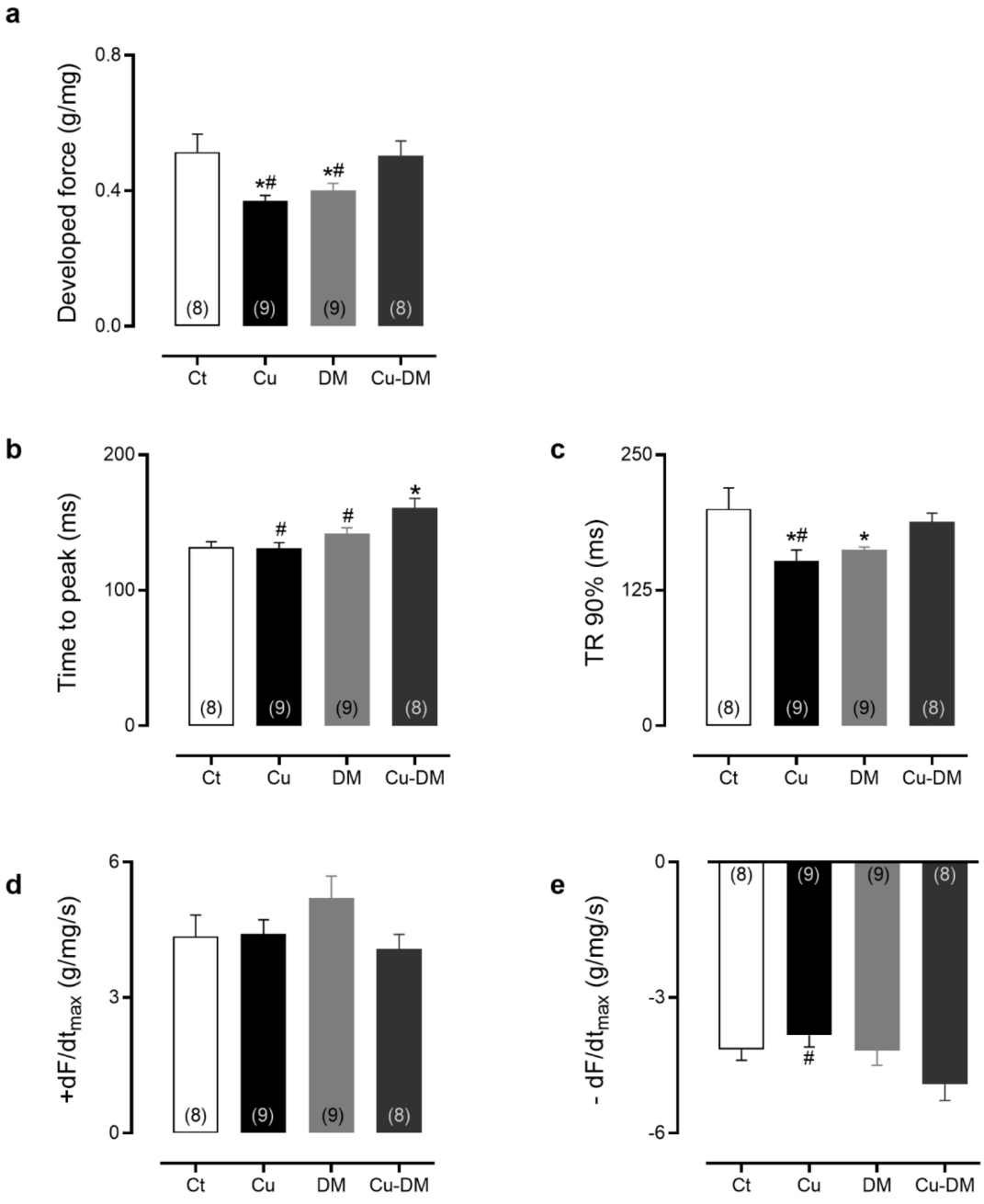


Figure 1

The *in vitro* myocardial mechanics was assessed as developed force normalized by respective muscle weight (a), time to peak of tension (b), time to 90% of Relaxation (c), its first time-derivative (+dF/dt_{max} and −dF/dt_{max}, d and e). Ct: control group; Cu: copper-exposed group; DM: diabetic group; Cu-DM: diabetic copper-exposed group (Cu-DM). Data are the means ± SEM. *P < 0.05 versus Ct, #P < 0.05

versus Cu-DM, using two-way ANOVA and Fisher's post hoc test. The number of individual biological subjects (animals) is indicated in the parentheses.

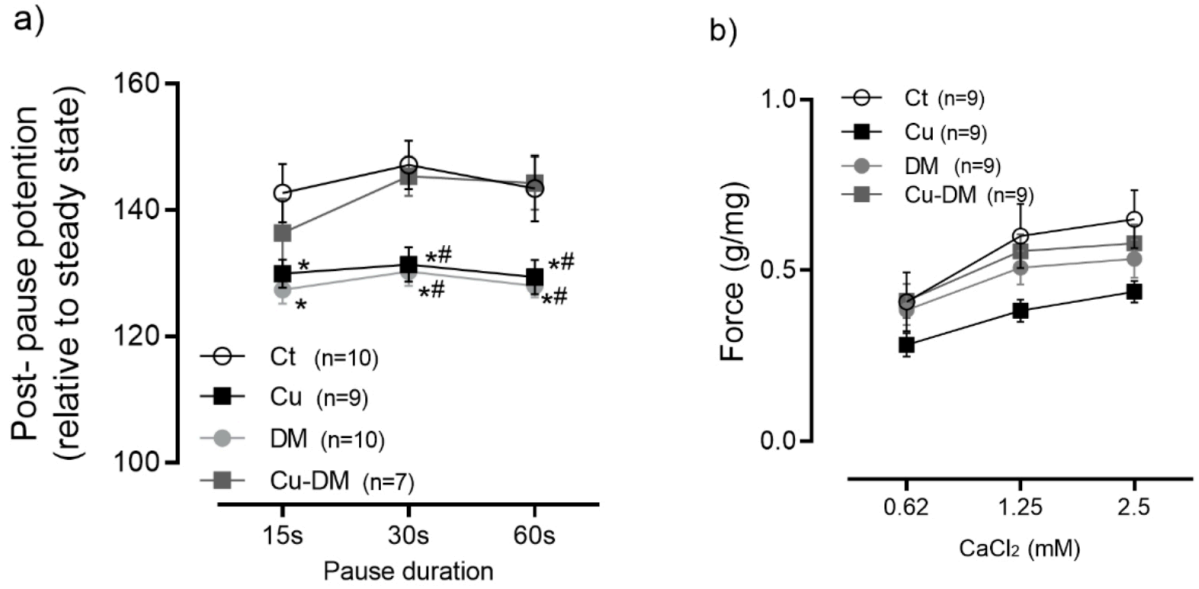


Figure 2

Contractile response to different experimental protocols: (a) Effect of increasing pause durations of the stimuli on the relative contraction developed by papillary muscles. Post-pause potentiation is expressed as a ratio between the contractions before and after the respective pause; (b) Developed force during increments of Ca^{2+} concentration at buffer solution. Ct: control group; Cu: copper-exposed group; DM: diabetic group; Cu-DM: diabetic copper-exposed group (Cu-DM). Bars and symbols represent means ± SEM. *P < 0.05 versus Ct, #P < 0.05 versus Cu-DM, using two-way ANOVA and Tukey post hoc test for multiple comparisons

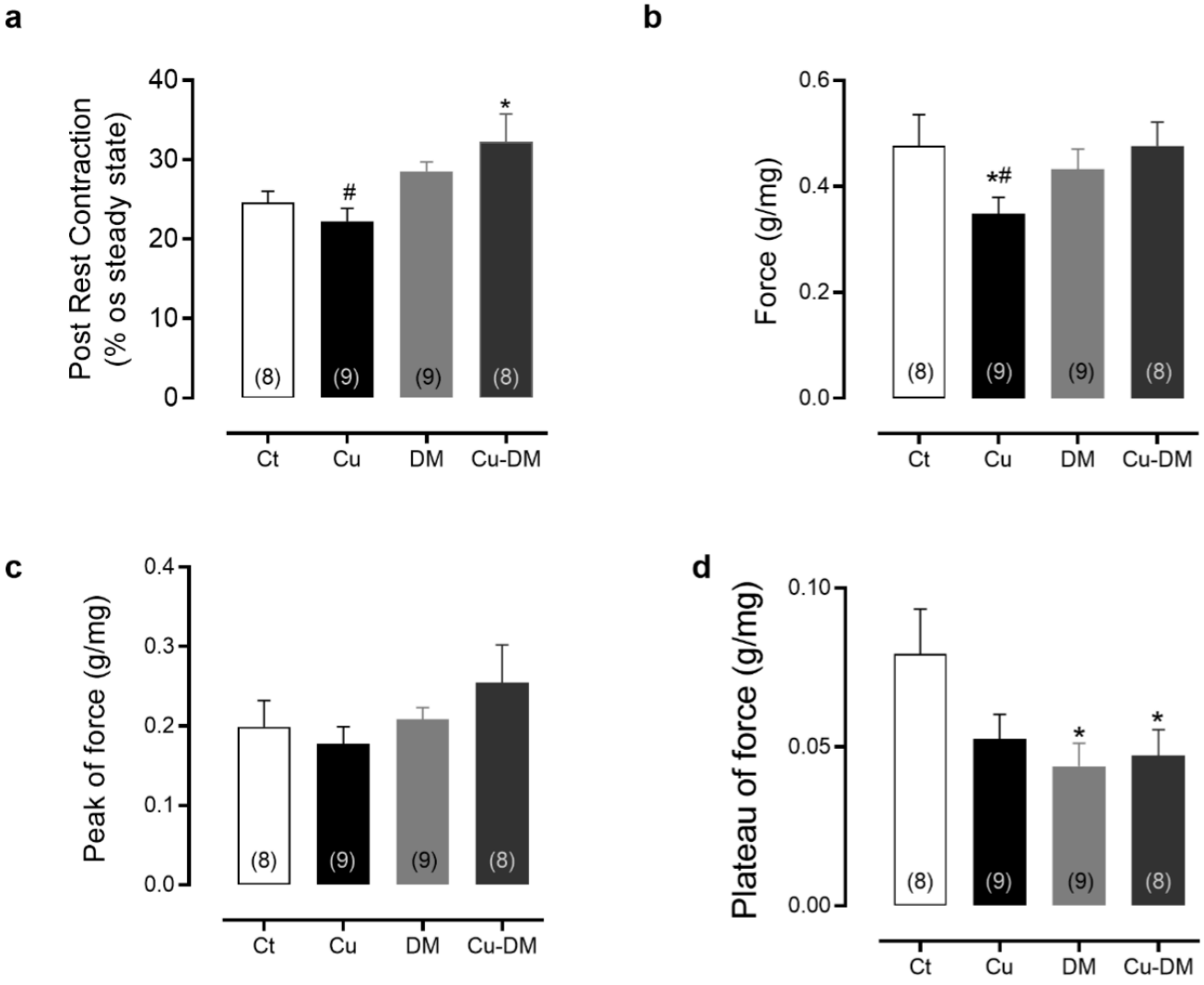


Figure 3

Contractile response to different experimental protocols: (a) Contraction dependent on transsarcolemmal calcium influx represented as the percentage of the respective steady state twitch; (b) Inotropic response of the cardiac muscle to isoproterenol (beta adrenergic agonist); (c) Relative tetanic peak force and (d) Relative tetanic plateau force in papillary muscles. Ct: control group; Cu: copper-exposed group; DM: diabetic group; Cu-DM: diabetic copper-exposed group (Cu-DM). Bars and symbols represent means ± SEM. *P < 0.05 versus Ct, #P < 0.05 versus Cu-DM, using one-way ANOVA and Fisher's post hoc test. The number of individual biological subjects (animals) is indicated in the parentheses

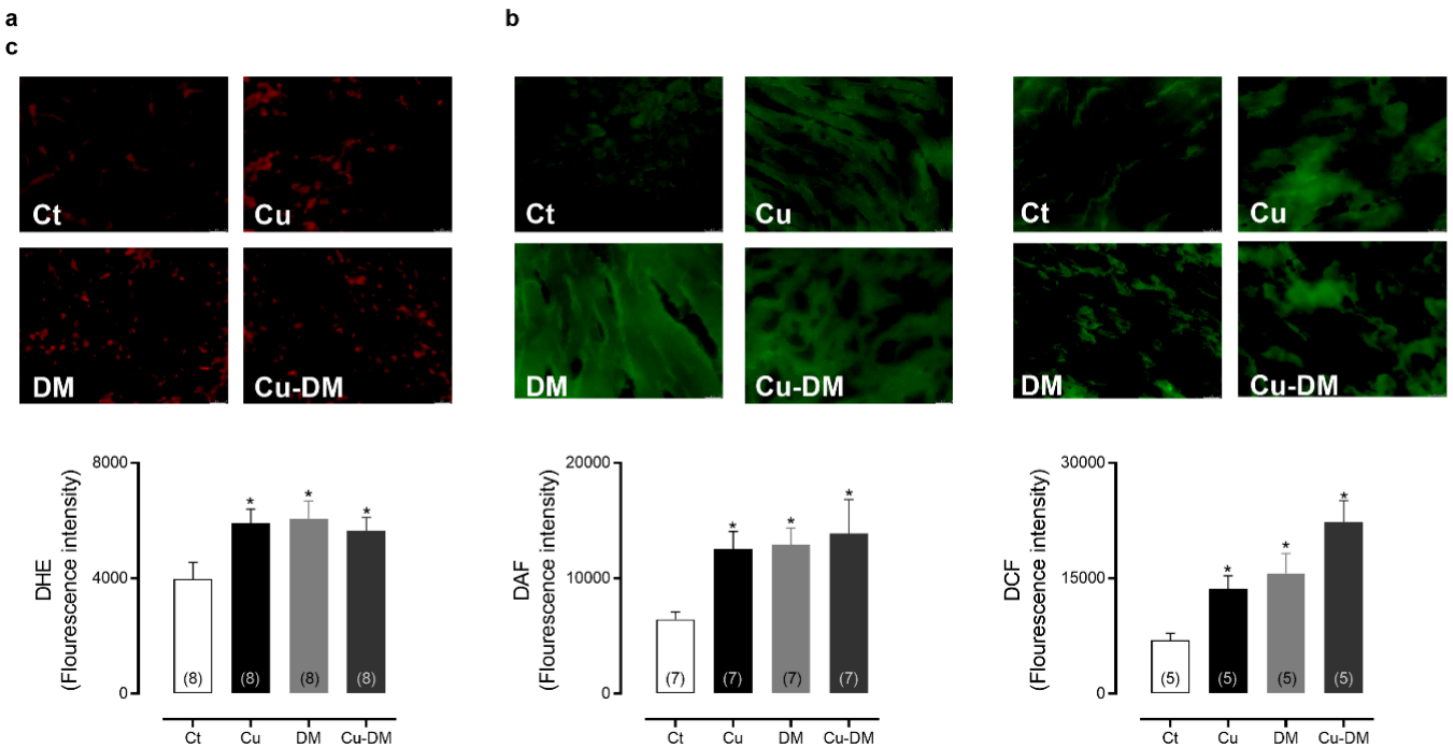


Figure 4

In situ measurements of superoxide anion, oxide nitric and hydrogen peroxide. Upper panels show representative fluorescent microphotographs of papillary muscle sections labeled with dihydroethidium (DHE), diaminofluorescein (DAF) dichlorofluorescein (DCF). Quantitative analysis of $O_2^{\bullet -}$ (a), NO (b) and H_2O_2 (c) production or bioavailability was also shown. Ct: control group; Cu: copper-exposed group; DM: diabetic group; Cu-DM: diabetic copper-exposed group (Cu-DM). Bars represent means ± SEM < 0.05 versus Ct, #P < 0.05 versus Cu-DM, using two-way ANOVA and Fisher's post hoc test. The number of individual biological subjects (animals) is indicated in the parentheses

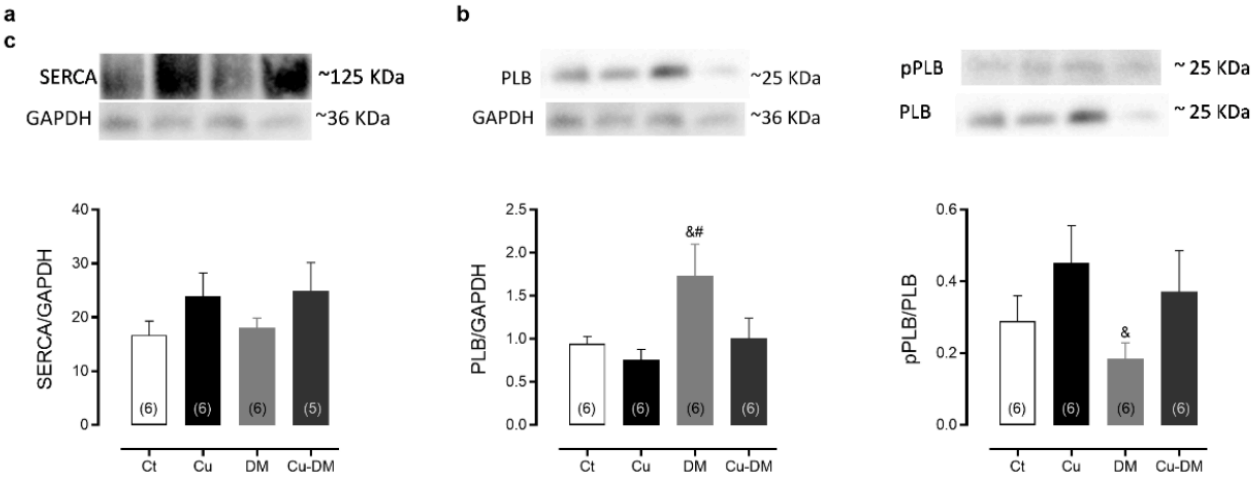


Figure 5

Protein expression analysis by Western blot of cardiac calcium-handling proteins. Expression of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) (a), phospholamban (PLB) (b), and phosphorylated phospholamban at serine 16 (pPLB) (c) in left ventricular papillary muscles.Ct: control group; Cu: copper-exposed group; DM: diabetic group; Cu-DM: diabetic copper-exposed group (Cu-DM). Bars represent means ± SEM. &P < 0.05 vs Cu; #P < 0.05 vs Cu-DM, using two-way ANOVA followed by Fisher's post hoc test. The number of animals is indicated in parentheses.