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ORGAN PHYSIOLOGY

Cobalt chloride postconditioning as myoprotective therapy in cardiac ischemia–reperfusion

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Abstract

Since damage induced by ischemia–reperfusion (I/R) involves alterations in Ca^{2+} homeostasis and is reduced by ischemic postconditioning (IP) and that $CoCl_2$ can trigger changes resembling the response to a hypoxic event in normoxia and its blockade on Ca^{2+} current in heart muscle, our aim was to evaluate $CoCl_2$ as an IP therapeutic tool. Mechanic and energetic parameters of isolated and arterially perfused male Wistar rat heart ventricles were simultaneously analyzed in a model of I/R in which 0.23 mmol/L $CoCl_2$ was introduced upon reperfusion and kept or withdrawn after 20 min or introduced after 20 min of reperfusion. The presence of $CoCl_2$ did not affect diastolic pressure but increased post-ischemic contractile recovery, which peaked at 20 min and decreased at the end of reperfusion. This decrease was prevented when $CoCl_2$ was removed at 20 min of reperfusion. Total heat release increased throughout reperfusion, while economy increased between 15 and 25 min. No effect was observed when $CoCl_2$ was introduced at 20 min of reperfusion. In addition, both the area under the contracture curve evoked by 10 mmol/L caffeine–36 mmol/L Na^+ and the contracture tension relaxation rate were higher with $CoCl_2$. Furthermore, $CoCl_2$ decreased the number of arrhythmias during reperfusion and the ventricular damaged area. The presence of $CoCl_2$ in reperfusion induces cardioprotection consistent with the improvement in cellular calcium handling. The use of $CoCl_2$ constitutes a potential cardioprotective tool of clinical relevance.

Keywords Postconditioning · Ischemia/reperfusion · Cobalt chloride · Heart · Myocardial infarction

Introduction

Myocardial ischemia occurs when blood flow to the heart is reduced or suspended reducing the supply of nutrients and oxygen to the cardiac muscle [21]. Cardiac dysfunction develops mainly during reperfusion, with reversible or irreversible functional myocardium damage depending on ischemia duration [5]. Ischemia–reperfusion (I/R) is one of the main cardiovascular risk factors and the underlying mechanism of diseases such as angina or heart attack. The heart depends on continuous metabolism to replenish ATP spent during the contraction-relaxation cycle, which makes energy balance essential. In all cases, ischemia causes a contractile and energetic dysfunction due to an imbalance between oxygen supply by coronary perfusion and myocardial demand dependent on frequency and contractility. Several factors appear to be involved in heart dysfunction or successful protection, including modifications in ionic homeostasis of H^+, Na^+, and Ca^{2+} [30] although the precise underlying mechanisms have not been fully elucidated yet.

Regarding energetic heart dysfunction evaluation, myothermal measurements can be used to study the heart metabolic status. Heat released by the cardiac muscle is related to ATP used in exothermic processes such as the active transport of ions, actomyosin interaction to produce force, and mitochondrial activities for recovery of the chemical energy used in each contraction–relaxation cycle. With increasing emphasis on the pivotal role of reperfusion on heart contractile recovery upon ischemia, several studies explored whether interventions during reperfusion could limit heart

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damage. Indeed, intermittent short repetitive interruptions of reperfusion at the very onset (ischemic postconditioning (IP)) were shown to provide protection in dogs, rats, and humans [19]. This is of great potential clinical significance, as ischemic lesions are seldom predictable.

The clinical translation of IP poses the risk of applying ischemic events to an organ that has undergone a previous severe ischemia. For this reason, it would be ideal to find drugs that mimic a brief ischemia or share common mechanisms with IP. In this sense and searching for strategies that mitigate damage caused by I/R, $CoCl_2$ has demonstrated to possess the necessary properties to function as a postconditioning element, as it can trigger transcriptional changes which resemble the response to a hypoxic event even in normoxic conditions. This response is characterized by an increase in both HIF-1α protein content and DNA binding activity, which leads to an increase in the expression of its target genes [20]. Furthermore, it is known that Co^{2+} acts blocking Ca^{2+} current in both skeletal and heart muscle as it blocks L–type Ca^{2+} channel and inhibits the Na^{+}-Ca^{2+} exchanger [7, 13, 39]. This drug has already been shown to act as an effective preconditioning agent [41, 42], although its cardioprotective activity when administered as IP treatment has not been evaluated yet. For this reason, the aim of this work is to evaluate $CoCl_2$ as a possible IP therapeutic tool in a model of cardiac I/R in isolated organs by simultaneous analysis of the mechanical and energetic parameters of heart muscle.

Materials and methods

Animals

Male Wistar rats were maintained on a 12:12 h light/dark cycle in a temperature- (21 ± 2 °C) and humidity-controlled (65 ± 5%) environment. Animals had access to food and tap water ad libitum.

Experimental procedure

Two-month-old rats of 260–300 g weight were euthanized with thiopental overdose (60 mg/kg i.p.) in the presence of heparin (2000 IU i.p), and the hearts were removed.

Heart treatment

The heart was arterially perfused with control Krebs solution (in mmol/L: 1 $MgCl_2$, 120 NaCl, 0.5 NaH_2PO_4, 7 KCl, 25 $NaHCO_3$, 1.35 $CaCl_2$ and 8 dextrose, bubbled with a gas mixture 95% O_2 and 5% CO_2) at a flow of 6 ml/min/g at 37 °C according to the Langendorff technique [11]. The atria were removed, and the focus of spontaneous contraction was suppressed by cutting the septum next to the aorta. A latex balloon of adjustable volume was introduced in the left ventricle and connected through a cannula to a pressure transducer Statham P23Db (Gould-Statham Instruments, Hato Rey, Puerto Rico). The continuously perfused heart was mounted in the inner chamber of a calorimetric flow system [29] which was immersed in a water bath at 37 ± 0.01 °C. The heart was electrically stimulated with pulses of 5 V and 5 ms at 3 Hz with two punctate electrodes connected to a Grass Model SD9 stimulator (Braintree, MA, USA). Calorimetric and mechanical signals were amplified and recorded continuously and simultaneously on a Grass polygraph Model 7 (Braintree, MA, USA) and digitized using an A/D converter (TL-1 DMA; Axon Instruments, Foster City, CA, USA). From the left ventricular pressure record, the maximum pressure developed during the isovolumetric contraction at optimum volume (P), the left ventricular end diastolic pressure (LVEDP), and the maximum speed of contraction (+P) and relaxation (−P) were calculated. Total heat release (Ht) (in mW/g wet mass) was continuously obtained in the presence or absence of perfusion throughout the I/R protocol. Calorimeter base line was obtained as previously indicated [6]. Total muscular economy was calculated as the ratio P/Ht (in mmHg/mW/g). In order to compare the post-ischemic recovery between protocols, all parameters were expressed as a percentage of the initial pre-ischemic values.

Ischemia–reperfusion protocol After 50-min stabilization in Krebs solution (control), heart was subjected to a 30-min ischemic challenge followed by 45-min reperfusion which was made in the presence or absence of 0.23 mmol/L $CoCl_2$ (Sigma Aldrich), or in the presence of $CoCl_2$ for 20 min and subsequent withdrawal. Furthermore, the introduction of 0.23 mmol/L $CoCl_2$ after 20 min of reperfusion period was evaluated. In another set of experiments after reperfusion with either control Krebs or Krebs containing $CoCl_2$ for the first 20 min, hearts were perfused with a low sodium–caffeine solution (in mmol/L: 1 $MgCl_2$, 11 NaCl, 0.5 NaH_2PO_4, 7 KCl, 25 $NaHCO_3$, 1.35 $CaCl_2$, 8 dextrose, 218 sucrose, and 10 caffeine) to induce contracture.

Normoxia protocol After 50-min stabilization in Krebs solution, heart was perfused with 0.23 mmol/L $CoCl_2$ for 30 min and subsequently washed out with Krebs solution for another 30 min. Heart contractile and energetic parameters were obtained at the end of the equilibration period (control), at 30 min of $CoCl_2$ treatment and at 30 min of $CoCl_2$ withdrawal.

Arrhythmias quantification A specific algorithm was developed using Python programming language to count reperfusion arrhythmias in each pressure record. It is based on distinguishing significant peaks which define the contraction frequency. Then, an arrhythmia is counted if an extra peak

higher than 5% of the total pressure is detected. When the signal is considerably irregular, the number of arrhythmias is counted as the number of abnormal contraction present in that period.

Infarct area quantification After the end of 90 min of reperfusion ventricles were immediately froze and cut in 1 mm slides and then incubated for 20 min at 37 °C in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffered saline (PBS), pH 7.8 and then washed twice with PBS and fixed in 10% formaldehyde for 24 h. Finally, digital images were obtained and analyzed using computerized planimetry (Image Analyzer, Image-Pro Plus). Infarct size was expressed as a percentage of the total ventricle area.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). The comparison between treatments was performed using ANOVA test for one or two factors as appropriate and Tukey test for post hoc analysis. When comparing two groups *t* test or Mann–Whitney test were used. Linear regression curves were calculated to determine the rate of contracture decline. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the Graphpad V.4 Prism software (San Diego, La Jolla, CA, USA).

Results

Effects of $CoCl_2$ added at the beginning of reperfusion

In order to analyze the effect of $CoCl_2$ during heart reperfusion upon an ischemic insult, simultaneous contractile and energy parameters were measured in ventricles isolated from rats (Fig. 1). LVEDP in pre-ischemic conditions rendered 13.52 ± 1.81 mm Hg. As shown in Fig. 1A in reperfusion the presence of $CoCl_2$ did not alter LVEDP. Regarding P (Fig. 1B), after ischemia, P gradually recovered reaching 30.1 ± 5.2% of the original value at the end. The presence of $CoCl_2$ during reperfusion caused a significant increase in P, which gradually rose and peaked at 51.8 ± 6.3% after 20 min. In the subsequent 25 min, P went down to reach the same level as controls at the end of reperfusion. This decrease was avoided when $CoCl_2$ was removed at 20 min of reperfusion, consequently P remained above control P values (Fig. 1B). As shown in Fig. 1C during reperfusion, Ht climbed to a maximum value of 66.0 ± 2.6% and gradually decreased to a final 53.6 ± 4.4%. The presence of $CoCl_2$ in reperfusion induced a significant increase in Ht, which peaked at 86.7 ± 5.1% and decreased to 69.4 ± 3.0% at the end of reperfusion (Fig. 1C). Contractile economy under pre-ischemic conditions was 3.7 ± 0.3 mmHg/(mW/g). During reperfusion, the presence of $CoCl_2$ significantly increased economy between 15 and 25 min and then decreased to reach

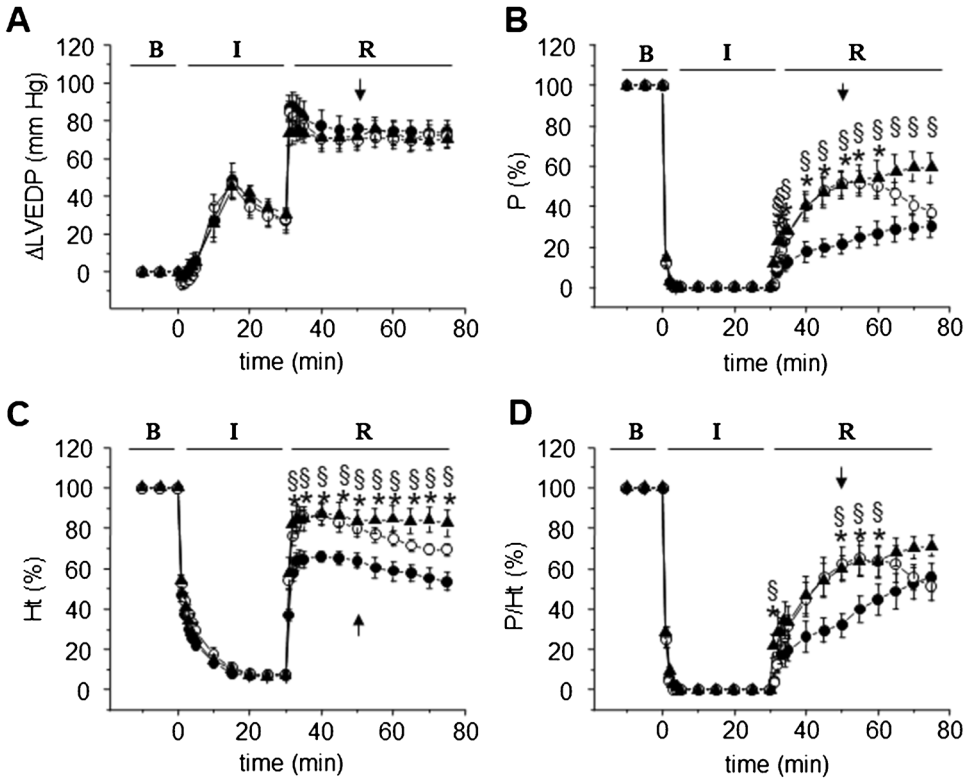

Fig. 1 Effect of $CoCl_2$ added at the beginning of reperfusion on heart mechanical and energetic parameters during I/R. Changes in end diastolic pressure (ΔLVEDP) (**A**), percentage of intraventricular pressure developed during contraction (P) (**B**), percentage of total heat flow (Ht) (**C**), and percentage of total muscle economy (P/Ht) (**D**) measured in rat hearts exposed to I/R in the presence (O) or absence of $CoCl_2$ (●) during reperfusion, or in the presence of $CoCl_2$ during reperfusion for 20 min and subsequent withdrawal (▲). Black arrow indicates the time of $CoCl_2$ withdrawal. B: basal, I: ischemia, R: reperfusion. Data are expressed as the mean ± SEM. Control: $n = 5$, $CoCl_2$: $n = 6$, $CoCl_2$ withdrawal: $n = 6$. Two-way ANOVA for repeated measurements**:** * $p < 0.05$ $CoCl_2$ vs. control, § $p < 0.05$ $CoCl_2$ withdrawal vs. control

control-like values at the end of reperfusion (Fig. 1D). This decrease was avoided when $CoCl_2$ was removed at 20 min of reperfusion (Fig. 1D).

Effect of $CoCl_2$ on normoxia

The fact that $CoCl_2$ effects appear quickly on reperfusion lead us to think that $CoCl_2$ action may be due to its cell calcium entry blocking activity [7, 13]. Then we tested $CoCl_2$ effects under normoxic conditions. As expected, $CoCl_2$ induced a negative inotropic effect, with a decline in P (between 38 and 50%) at 30 min of perfusion. $CoCl_2$ withdrawal restored P to values between 85 and 91% of the pre-treatment ones (Table 1). Consistently, in the presence of $CoCl_2$, Ht decreased and recovered after $CoCl_2$ removal (Table 1). Similar behavior was observed in total contractile economy (P/Ht) (Table 1).

Velocities

Since $CoCl_2$ had a positive inotropic effect during reperfusion upon an ischemic episode (Fig. 1B), possible alterations in cell Ca^{2+} handling were considered. For this reason, the + P/P and − P/P ratios at 20 min of reperfusion were analyzed as an indirect measure of Ca^{2+} release and reuptake dynamics, respectively, during a beat. No differences were observed in both ratios between control and $CoCl_2$-treated hearts (% + P/P: 95.1 ± 8.2, n: 5 and 93.7 ± 1.0, n: 6, for control and $CoCl_2$, respectively; % − P/P: 103.6 ± 3.7, n: 5 and 104.7 ± 5.9, n: 6, for control and $CoCl_2$, respectively), which may indicate that $CoCl_2$ does not effectively affect twitch Ca^{2+} release or reuptake mechanisms during reperfusion.

Arrhythmias

Due to the fact that reperfusion causes a cell Ca^{2+} overload and that an excess in Ca^{2+} cycling in and out of sarcoplasmic reticulum (SR) increases the number of ventricular extrasystoles [31], the incidence of reperfusion arrhythmias was evaluated. The presence of $CoCl_2$ significantly decreased the incidence of arrhythmias evaluated between 10 and 20 min of reperfusion (Fig. 2). Of note, it was checked that signals

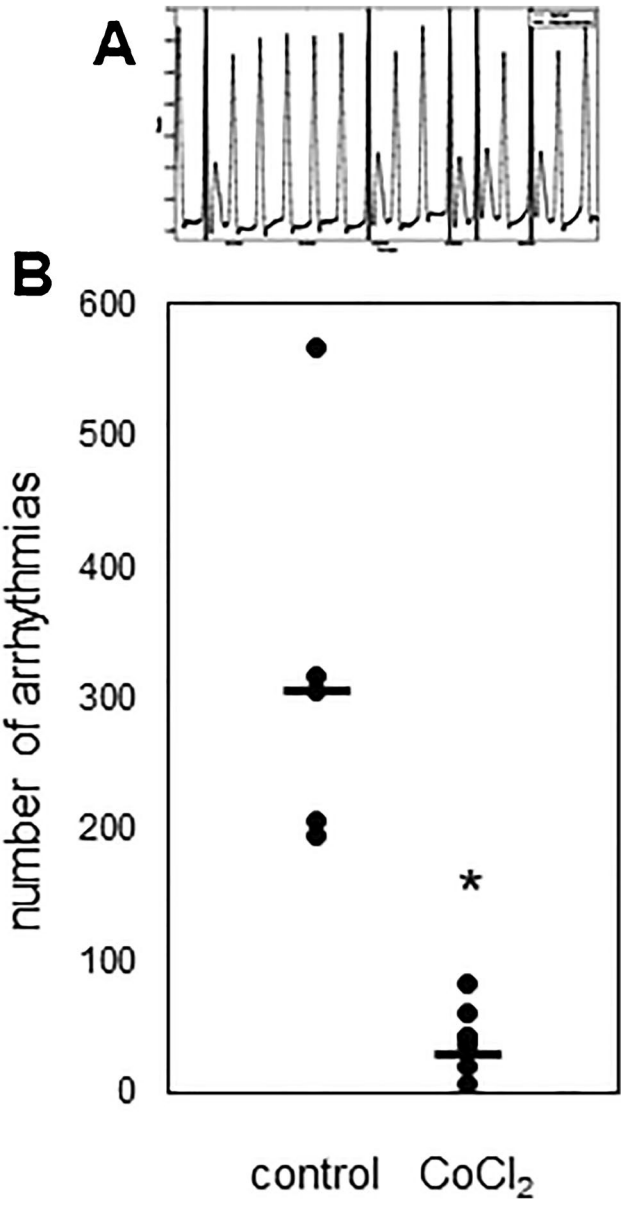

Fig. 2 Arrhythmias. **A** Representative software image where dark bars indicate the presence of arrhythmia. B: Quantification of cardiac arrhythmias produced between 10 and 20 min of reperfusion period in the presence or absence of $CoCl_2$. Bars represent the mean value of each group. Control: $n = 5$, $CoCl_2$: $n = 7$. Mann–Whitney test: * $p < 0.05$ vs. control

Table 1 Effects of $CoCl_2$ under normoxia. Heart contractile and energetic parameters obtained at the end of the equilibration period (control), $CoCl_2$ treatment and upon $CoCl_2$ withdrawal (30 min each). Data are expressed as the mean ± SEM of three experiments. Kruskal–Wallis test. *$p < 0.05$ vs control

	Perfusion P (mmHg)	LVEDP (mmHg)	P (mmHg)	+ dP/dt (mmHg/s)	-dP/dt (mmHg/s)	Ht (mW/g)	P/Ht mmHg/(mW/g)
Control	113.7 ± 17.2	8.73 ± 3.78	72.54 ± 8.85	1844.0 ± 87.8	1246.9 ± 72.5	20.3 ± 2.6	3.59 ± 0.14
$CoCl_2$ treatment	164.8 ± 14.3	25.58 ± 4.41*	32.13 ± 4.41*	809.3 ± 155.9*	509.5 ± 99.7*	12.7 ± 0.7*	2.58 ± 0.51
$CoCl_2$ withdrawal	146.0 ± 10.8	32.80 ± 6.58*	63.70 ± 6.45	1551.2 ± 42.5*	1045.0 ± 26.7*	15.6 ± 1.0	4.08 ± 0.34

measured as arrhythmias were not due to the alternance phenomenon.

Evaluation of cardiac damage area

In another set of experiments, the cardiac damaged area was evaluated by TTC staining after ischemia when reperfusion was made (Fig. 3A).

As shown in Fig. 3B, a reduction in the infarcted area was observed in the ischemic ventricles in which $CoCl_2$ was present in the reperfusion for 20 min and then removed.

Caffeine–low Na^+–induced contracture

Since SR is the organelle responsible for Ca^{2+} signal amplification for contraction and the presence of $CoCl_2$ during reperfusion induced positive inotropism, the SR Ca^{2+} content should be increased. Contracture by caffeine and low Na^+ is an indirect way of evaluating Ca^{2+} content in the SR, as caffeine can empty this organelle of its Ca^{2+}, and a low Na^+ medium prevents Ca^{2+}exit from the cell [28]. In our study, the presence of $CoCl_2$ rendered higher contracture pressure induced by caffeine–low Na^+ media (Fig. 4A), a larger area under the contracture curve (Fig. 4B), and also a higher rate of contracture tension relaxation (Fig. 4C).

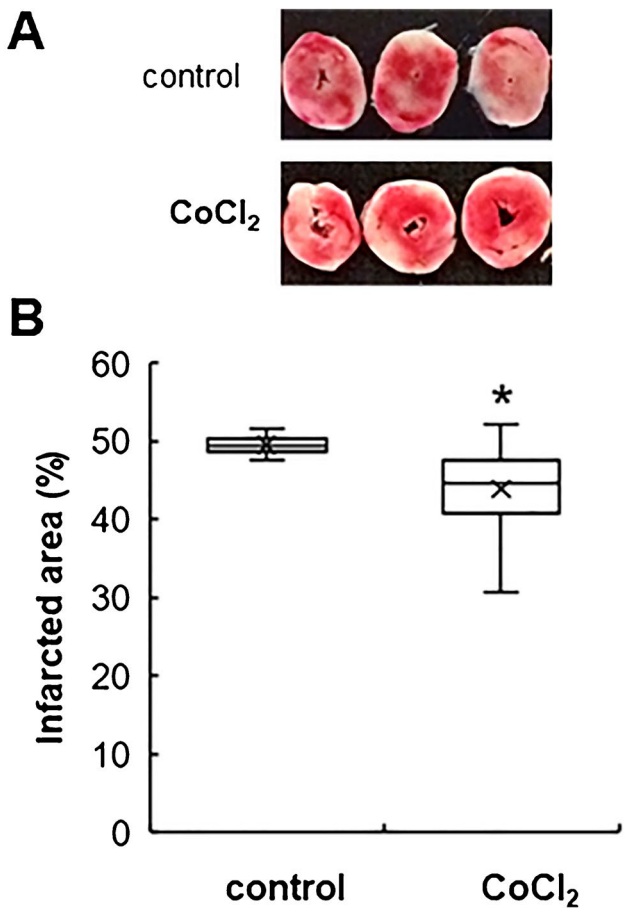

Fig. 3 Myocardial Infarction Area. **A** Representative images of the heart sections dyed with TTC. **B** Quantification of the infarct size area. Bars represent the mean value of each group. Mann–Whitney test $*p < 0.05$ vs control

Effects of $CoCl_2$ added at 20 min of reperfusion

To evaluate whether the improvement in mechanical and energetic parameters induced by $CoCl_2$ involves the prevention of the expected calcium overload at the beginning of reperfusion, $CoCl_2$ was added at 20 min later. As shown in Fig. 5, the cardioprotective action of $CoCl_2$ is lost when administered later in reperfusion, as no alterations were observed in mechanical (Fig. 5 A and B) or energetic parameters (Fig. 5 C and D).

Discussion

Cardiac I/R injury involves a series of pathological processes in which energetic state, mechanical function, and tissue survival are compromised. Finding both pre- and post-ischemic protective strategies is relevant to mitigate damage occurring primarily during tissue reperfusion. In this sense, to our knowledge, this work is the first to evaluate $CoCl_2$ as a possible post-conditioning tool, analyzing its effect on cardiac mechanics and energy in an experimental model of isolated ventricles exposed to I/R, which is a widely validated method for evaluation of the direct effects of medications on heart function [40]. $CoCl_2$ has been extensively used in clinical practice without appreciable toxic effects as effective treatment for anemia, for infants and patients undergoing hemodialysis [12], as it is a well-established chemical inducer of in vivo hypoxia-like responses such as an increase in erythropoiesis and angiogenesis. For the same reasons, $CoCl_2$ is also administered to improve athletic performance [22]. $CoCl_2$ is also considered not to have significant toxic effects at as much as 40 mg/kg oral dose for up to 4 months, although toxicity has been reported at longer exposure or excessive administration and in this case it was related with cardiomyopathy [2, 3]. This work used 0.23 mmol/L $CoCl_2$, which is equivalent to the serum concentration reached by a single intraperitoneal administration of 20 mg/kg used in preconditioning studies [32]. Consequently, no adverse effects are expected. In concordance with this hypothesis, Shrivastava et al. demonstrated that cobalt is nontoxic and may therefore be considered for developing a suitable neutraceutic product [35]. It was previously observed that IP produces ischemic tolerance, although the underlying molecular mechanisms are still unclear. The precise mechanism by which $CoCl_2$ produces a hypoxia-like response is not fully understood yet, although several

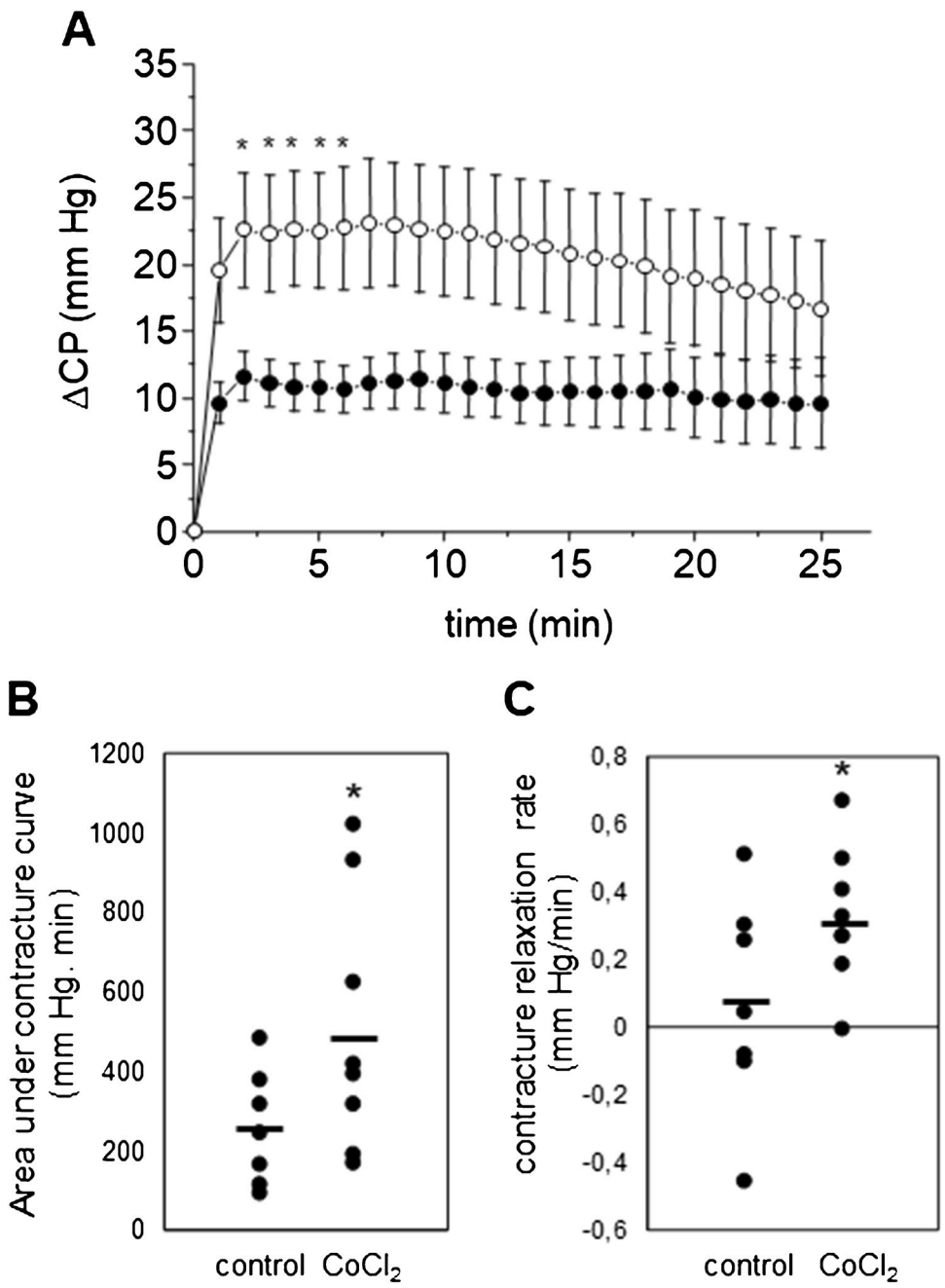

Fig. 4 Contracture induced by caffeine-low Na^+ media. Changes in contracture pressure (ΔCP) above the LVEDP of immediately previous beating heart induced by caffeine and low Na^+ media in hearts perfused for 20 min in the presence (O) or absence of $CoCl_2$ (●) during reperfusion (**A**). Area under the curve of contracture (**B**) and the rate of contracture tension relaxation (**C**). Bars represent the mean value of each group. Control: $n=7$, $CoCl_2$: $n=8$. *T* test: $*p<0.05$ vs. control

hypotheses involve an increase in the transcription factor HIF-1α. Co^{2+} may increase HIF-1α activity by several mechanisms [10, 17, 20, 44] and also may act by HIF-1α -independent pathways [26]. HIF-1α can regulate the expression of several proteins involved in the cardioprotective effect of $CoCl_2$ [33] such as iNOS, involved in the delayed myocardial cardioprotection [4]. Of note, the increase of HIF-1α by $CoCl_2$ has been detected by western blot at times greater than 1 h in various systems [9, 24, 43]. Although we do not rule out that its activity may manifest at shorter times, the participation of HIF-1α-induced proteins such as iNOS in the cardioprotective effect observed in our study seems unlikely. It is well known that $CoCl_2$ blocks L -type Ca^{2+} channel in both skeletal and heart muscle [7, 13] and also acts as an inhibitor of the Na^+-Ca^{2+} exchanger [39]. In our work, cardioprotection was detected when $CoCl_2$ was introduced during reperfusion. Given the relatively short period of time in which this process occurs and the fact that some effects are abolished by $CoCl_2$ withdrawal, we consider that the blocking action on Ca^{2+} channels and, mainly, the inhibitory effect on Na^+-Ca^{2+} exchanger could be the main responsible for $CoCl_2$ effect on post-ischemic recovery. When exposed to ischemia, the cardiac muscle suffers an energetic misbalance and hence an ionic one. As inhibition of Na^+/K^+

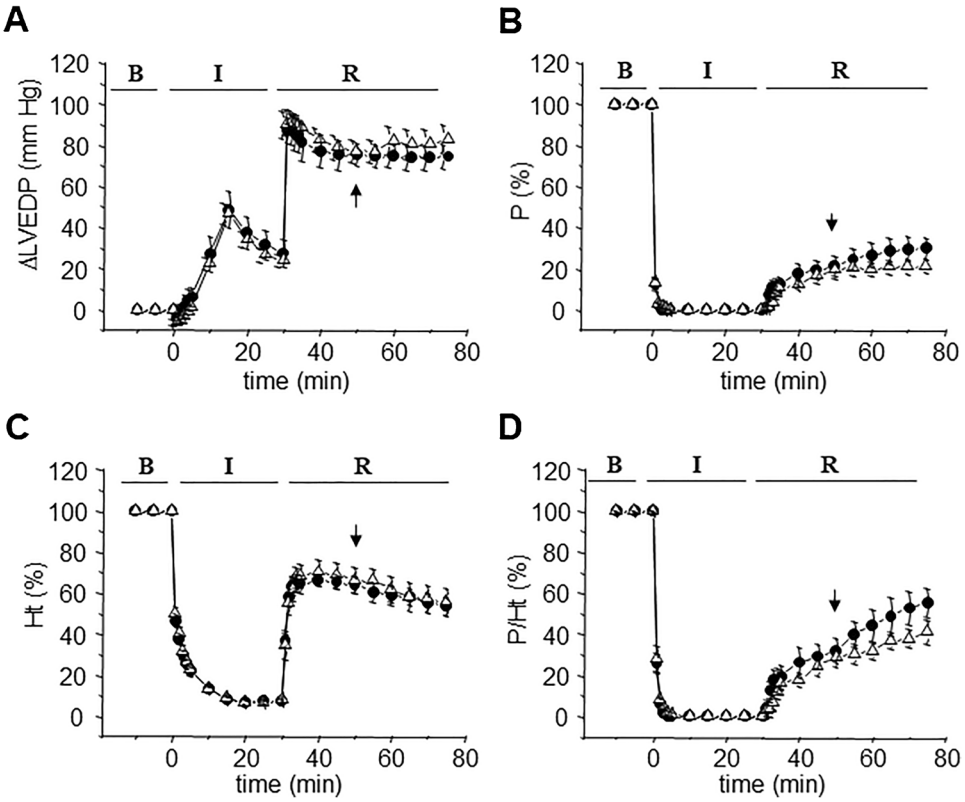

Fig. 5 Effect of $CoCl_2$ added later during reperfusion on heart mechanical and energetic parameters during I/R. Changes in diastolic pressure (ΔLVEDP) (**A**), percentage of intraventricular pressure developed during contraction (P) (**B**), percentage of total heat flow (Ht) (**C**) and percentage of total muscle economy (P/Ht) (**D**) measured in rat hearts exposed to I/R in the absence of $CoCl_2$ (●) or in the presence of $CoCl_2$ added at 20 min of reperfusion. Black arrow indicates the time of $CoCl_2$ addition. B: basal, I: ischemia, R: reperfusion. Data are expressed as the mean ± SEM. Control: $n = 5$, $CoCl_2$: $n = 7$. Two-way ANOVA for repeated measurements. No significant differences vs control

ATPase progresses, the cardiac muscle increases contracture tension, which has been associated with cytosolic Na^+ overload leading to Ca^{2+} overload, where the Na^+-Ca^{2+} exchanger plays a key role [25]. Increased contracture tension has also been connected with energy deficits caused by the failure of ATP synthesis and mitochondrial uncoupling [18, 23]. This situation worsens during reperfusion, which causes both cytosolic and mitochondrial Ca^{2+} overload and an increase in reactive oxygen species [16, 17]. In this phase, contractile recovery is slow but improves as energetic balance upgrade occurs, leading to a partial reversion of Ca^{2+} overload. In the presence of $CoCl_2$, the contractile force evaluated through developed pressure (P) showed a substantial increase until 20 min of reperfusion. This finding indicates improved mechanical performance, in agreement with its Ca^{2+} entry inhibitor activity, which might protect cells from Ca^{2+} overload. This cardioprotection is transient, as P decreases at longer times of $CoCl_2$ exposure, which means that the negative inotropic effect of Co^{2+} prevails at prolonged times. When Co^{2+} is removed from perfusion media at the time of maximal cardioprotection, the negative inotropic effect is not observed, also indicating that this effect is reversible. In fact, the presence of $CoCl_2$ in normoxic conditions evidences the negative inotropic effect which is reversed when $CoCl_2$ is removed. In contrast, this work shows that contracture tension evaluated through changes in diastolic pressure (ΔLVEDP) exhibit a control-like pattern in the presence of $CoCl_2$ during reperfusion, indicating that $CoCl_2$ does not affect post-ischemic diastolic function. In cardiac muscle, metabolic activity can be evaluated through the measurement of heat production during contractile activity. Total metabolic activity (Ht) is a reflection of all processes taking place during the cardiac excitation–contraction-relaxation cycle [6]. In the present work we observed an increment in P accompanied by an increase in Ht in the presence of $CoCl_2$ during reperfusion. However, the increase observed in Ht cannot be single-handedly explained by P, as they did not increase proportionally. In fact, P/Ht as an indicator of contraction economy also improved, which suggests that muscle is metabolically more efficient when $CoCl_2$ is present during reperfusion. The mechanical and energetic results obtained allow us to hypothesize that, at least in part, $CoCl_2$ protects the heart injured by I/R through better cellular Ca^{2+} handling, by ameliorating cellular Ca^{2+} overload, and hence preventing mitochondrial uncoupling during reperfusion. This hypothesis is further supported by the improvement in contractile economy obtained in the presence of $CoCl_2$ and the higher rate of contracture tension relaxation by caffeine and low Na^+ media when $CoCl_2$ was present. Of note, in this situation an increased velocity of relaxation is observed in the presence of $CoCl_2$ which could be related to an enhanced mitochondrial participation in calcium uptake as a parameter of better mitochondrial health. In addition, the fact that cardioprotection was not observed

when $CoCl_2$ was added at 20 min of reperfusion reinforces the hypothesis that $CoCl_2$ exerts its protective action by preventing Ca^{2+}overload, which occurs at the beginning of reperfusion. Furthermore, the decrease observed in the number of arrhythmias by reperfusion in the presence of $CoCl_2$ reflects a reduction in Ca^{2+} overload in the SR [30, 31], which further supports the hypothesis stated above. The cardioprotection was also observed as a reduction in the infarcted area. Although the effects of $CoCl_2$ observed in this work can be explained partially by its action on Ca^{2+} homeostasis, other explanations should not be ruled out, as Co^{2+} penetrates the cells [34] and may compete for intracellular Ca^{2+} binding proteins and thus exert inhibitory effects on Ca^{2+} signaling [1]. Several actions of $CoCl_2$ exposure have been described. Thus, at the mitochondrial level, it has been shown that $CoCl_2$ induces apoptosis, inhibition of both the ATP synthesis and electron transport as well as induction of mitochondrial permeability transition pore (mPTP) opening leading to severe mitochondrial dysfunction [8, 37]. The levels of Co^{2+} necessary to affect mitochondrial functions ranged between 5 and 100 µM [8]. However the intracellular concentration of Co^{2+} estimated from 25 min of extracellular exposure at 0.23 mM $CoCl_2$, both conditions used in this work, and at physiological calcium concentrations would rise to a maximum of 1.7 µM [36]. This Co^{2+} level is below the concentration reported to produce mitochondrial dysfunction and although it cannot be totally rule out Co^{2+} effects, if any, would be marginal. Furthermore, although apoptosis has been reported in the presence of Co^{2+} concentrations at the order of those used in this work, it only becomes evident in prolonged treatments (greater than 6 h), which is far from the exposure times used in this work [37, 38]. On the other hand, ROS production in the presence of Co^{2+} concentrations such as those used in this work reach similar levels to those caused by severe hypoxia [10]. Since ROS generation comes from different sources, i. e. cytosolic in the Co^{2+} exposure and mitochondrial in severe hypoxia, it could be argued that the presence of Co^{2+} during reperfusion could add enough ROS that would lead to exhausting antioxidants tissue reserves and thus impair post-ischemic recovery. The fact that this is not evident in our experimental conditions would suggest the preponderance of some other mechanisms such as the Co^{2+}- participation in the improvement of post-ischemic recovery previously hypothesized. The use of $CoCl_2$ as a cardioprotector in rats has been previously reported by Endoh et al. [14], as the authors observed improved cardiac contractile function in rats suffering hypoxia when previously administered water containing 0.01% $CoCl_2$ for 6–7 weeks. Although these studies were developed in different conditions—i.e. preconditioning treatment and hypoxia—and even if our current results cannot be directly extrapolated to animals suffering cardiac ischemia, both reports appear to pave the way for considering treatment with $CoCl_2$ during reperfusion an effective therapy to treat the damage produced by I/R. In conclusion, altogether, the results obtained in this work indicate that the presence of $CoCl_2$ in reperfusion induces cardioprotection by improving cellular Ca^{2+} handling. The search for therapies that allow mitigating the damage caused by ischemia reperfusion is a goal of clinical importance. In fact, in certain situations found in medical practice such as angina, heart attack, or surgical situations such as stent placement, aorto-coronary bypass, or heart transplantation, it is necessary to re-infuse the myocardium in order to rescue it from the previous period of ischemia. In these situations, the application of Co^{2+} during the first minutes of reperfusion could contribute to a better recovery of cardiac function. Therefore, the use of $CoCl_2$ constitutes a potential economical cardioprotective tool of clinical relevance.

Author contribution Conception or design of the work: Castilla, R and Bonazzola, P. Acquisition, analysis, or interpretation of data for the work: all authors. Drafting of the work or revising it critically for important intellectual content: Castilla, R; Fernández Vivanco, M; and Bonazzola, P.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Animal welfare All procedures involving animals were approved by the Institutional Committee of Animal Care and Use at the University of Buenos Aires (CICUAL, School of Medicine) and conducted according to the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 2011).

Conflict of interest The authors declare competing interests.

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