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Sodium overload during postnatal phases impairs diastolic function and exacerbates reperfusion arrhythmias in adult rats

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Abstract

Sodium overload during childhood impairs baroreflex sensitivity and increases arterial blood pressure and heart rate in adulthood; these effects persist even after high-salt diet (HSD) withdrawal. However, the literature lacks details on the effects of HSD during postnatal phases on cardiac ischemia/reperfusion responses in adulthood. The current study aimed to elucidate the impact of HSD during infancy adolescence on isolated heart function and cardiac ischemia/reperfusion responses in adulthood. Male 21-day-old Wistar rats were treated for 60 days with hypertonic saline solution (NaCl; 0.3M; experimental group) or tap water (control group). Subsequently, both groups were maintained on a normal sodium diet for 30 days. Subsequently, the rats were euthanized, and their hearts were isolated and perfused according to the Langendorff technique. After 30 min of the basal period, the hearts were subjected to 20 min of anoxia, followed by 20 min of reperfusion. The basal contractile function was unaffected by HSD. However, HSD elevated the left ventricular end-diastolic pressure during reperfusion (23.1 ± 5.2 mmHg vs. 11.6 ± 1.4 mmHg; $p < 0.05$) and increased ectopic incidence period during reperfusion (208.8 ± 32.9s vs. 75.0 ± 7.8s; $p < 0.05$). In conclusion, sodium overload compromises cardiac function after reperfusion events, diminishes ventricular relaxation, and increases the severity of arrhythmias, suggesting a possible arrhythmogenic effect of HSD in the postnatal phases.

Background

Myocardial infarction, which is characterized by myocardial ischemia, is a leading cause of mortality globally.[1,2] The high mortality rate associated with this disorder can be partially attributed to the prevalence of ischemia/reperfusion events, which have deleterious effects on cardiac function.[3,4] Myocardial ischemia/reperfusion induces several types of ventricular arrhythmias such as ventricular premature/ectopic beats, ventricular tachycardia and ventricular fibrillation.[5] Arrhythmias are the major cause of death in the ischemia/reperfusion process, and can be caused by cardiac fibrosis.[6–8]

The harmful effects of a high-salt diet (HSD) during adulthood on arterial blood pressure (ABP) and cardiac function in humans and animal models have been previously demonstrated.[9,10] Indeed, spontaneously hypertensive rats exposed to HSD present diastolic dysfunction.[9,10] Additionally, a chronic increase in sodium intake is associated with deterioration in myocardial contractile function[11] and an elevated risk of mortality following myocardial infarction.[12,13] Changes in sodium/calcium homeostasis resulting from ischemia/reperfusion induce cardiac rhythm disturbances, including lethal ventricular arrhythmias and post-ischemic contractile dysfunction.[14,15]

Despite the well-characterized effects of sodium overload during adulthood, its impact of sodium overload during infancy and adolescence on cardiac function remains to be elucidated. Sodium consumption among children has been exacerbated by overconsumption of processed food products.[16,17] Elevated snack food intake among infants aged 9–11 months and toddlers aged 12–15 months is associated with high-sodium food consumption, affecting diet quality in

early childhood.[16] In addition, the association between excess sodium consumption and hypertension in children has been demonstrated.[18,19]

The developmental origins of the health and disease (DOHaD) hypothesis establishes a link between the early developmental phases and long-term cardiometabolic disorders.[20] Our research group has demonstrated that the postnatal period plays a key role in the development of cardiovascular diseases in adulthood.[21] Among the consequences in adulthood caused by sodium overload during childhood, it is noteworthy the diminished baroreflex sensitivity and diminished hypernatremia-induced renal vasodilation and increased mean ABP and heart rate (HR).[21] However, the effects of HSD during infancy and adolescence on ischemia/reperfusion-induced alterations in myocardial function have not yet been assessed. We hypothesized that HSD during infancy-adolescence affects cardiac function and increases the propensity for arrhythmia in adulthood. Thus, our study aimed to evaluate the effects of HSD during these developmental phases on left ventricular contractile function and cardiac reactivity to ischemia/reperfusion in the hearts of adult animals.

Materials and methods

Animals

All experiments were conducted on male Wistar rats aged twenty-one days old obtained from the central animal facility of the Federal University of Goiás. These animals were housed at the Department of Physiological Sciences in polypropylene cages ($45 \, cm \times 30 \, cm \times 15 \, cm$) under controlled conditions of 12-h light–dark cycle (07:00 a.m. – 07:00 p.m.), temperature (22–24°C), and free access to food (0.4% NaCl; AIN-93M)[22] and tap water or hypertonic saline solution (NaCl; 0.3M; Sigma-Aldrich, St Louis, MO, USA). All experimental procedures followed the rules and Guidelines for the Care and Use of Laboratory Animals approved by the Ethics Committee of the Federal University of Goiás (protocol number 023/15).

High-sodium diet protocol

Male Wistar rats were treated with HSD (NaCl; 0.3M; experimental group) or tap water (normal sodium diet; control group). The administration of HSD started on the 21st day of life and was completed after 60 days of treatment. After this period, tap water was offered to both groups throughout the subsequent 30 days (recovery period). Fluid and food were provided *ad libitum*.

Isolated rat hearts

At the end of the recovery period, the rats were decapitated 10–15 min after intraperitoneal injection of 200 IU heparin. Hearts were carefully dissected and perfused using the Langendorff technique. Briefly, the hearts were perfused through the aortic stump with Krebs-Ringer solution containing (in mmol/L) NaCl (118.4), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4 \cdot 7 \, H_2O$ (1.2), $CaCl_2 \cdot 2 \, H_2O$ (1.25), glucose (11.7), and $NaHCO_3$ (26.5). The perfusion flow was maintained at a constant rate (10 mL/min) at 37°C, with constant oxygenation (5% CO_2 and 95% O_2). A balloon was inserted into the left ventricle through the left atrium for isovolumetric recordings of left ventricular pressure (left ventricular end-systolic pressure, LVESP; left ventricular end-diastolic pressure, LVEDP; maximal rate of left ventricular pressure rise – +dP/dt; maximal rate of left ventricular pressure decline – − dP/dt;

perfusion pressure and left ventricular developed pressure – LVDevP, calculated as the difference between LVESP and LVEDP). Coronary perfusion pressure was measured using a pressure transducer connected to the aortic cannula and coupled to a data acquisition system (Dataq Instruments, Akron, OH, USA). After a stabilization period (30 min), the hearts were subjected to 20 min of anoxia (interruption of perfusion flow), followed by 20 min of reperfusion. The global ischemia/reperfusion Langendorff technique is widely used to mimic situations of acute myocardial infarction and cardiac arrest.[23,24] Reperfusion arrhythmias were defined as the presence of ventricular fibrillation and/or tachycardia during the reperfusion period. To obtain a quantitative measurement, the arrhythmias were classified by their total duration on a modified scale from a previous study.[25] The occurrence of cardiac arrhythmias for up to 1 min was attributed to factor 0; 1–4 min was attributed to factor 2; 4–8 min was attributed to factor 4; 8–12 min was attributed to factor 6; 12–16 min was attributed to factor 8; and 16–20 min was attributed to factor 10. A value from 0 to 10 was obtained in each experiment and was indicated as an arrhythmia severity index (ASI).

Histological analysis

Histological analyses were performed on the hearts of rats subjected to the same experimental protocol, except for the global ischemia/reperfusion. After the recovery period, the rats were decapitated, and the intermediate segments of the left ventricles were collected for histological analysis. To perform morphological analysis, the left ventricles were transversely divided into three portions, and intermediate portions were used. The segments were fixed in Metacarn solution (Supplementary Table S1) for 4 h. After fixation, the tissues were dehydrated in ethanol series (Neon®), cleared in xylene (Synth®) and embedded in paraffin (Sigma®; Supplementary Table S2). After inclusion, three 5 μm thickness sections (with 150 μm intervals) were obtained from each animal using a microtome (Leica RM2155, Nussloch, Germany). After cutting, the sections were fixed on glass slides (Exacta, $26 \times 76 \times 1 \times 1.2 \, mm$), rehydrated (Supplementary Table S3), and subjected to the cytochemistry technique using Gömori's reticulin (Supplementary Table S4). After staining, sections were dehydrated (Supplementary Table S5) and mounted on coverslips.

Morphometry of cardiomyocytes and stereology

The ventricular mass index (VMI), defined as the ratio between the left ventricle weight and tibia length,[26] was calculated for each animal to determine the ventricular hypertrophy level. Cardiomyocyte thickness (μm) of the left ventricles was measured to determine the degree of cellular hypertrophy. The transversal sections were photographed (at 400x magnification), and photomicrographs were used for morphological analysis and to measure cardiomyocyte thickness. The long-cross-sectional area (LCSA) of 200 cardiomyocytes from each group was measured. Only cells arranged longitudinally, with visible nuclei and cellular limits, were considered for analysis. The LCSA (μm) of each cardiomyocyte was measured in the region of the nucleus.

The relative frequency (%) of cardiomyotubules, non-fibrous connective tissue (which includes cells, ground substance, blood vessels, and nerves), and subtypes of collagen fibers was determined using the stereological method.[27] This method has already been validated for other tissues that also present randomly oriented and homogeneously distributed structures such as the lung,[27] placenta,[28] prostate[29] and cardiac muscle.[30] The analysis

was performed from a test system available in the image Pro-Plus 6.1 program for Windows (Media Cybernetics Inc., Silver Spring, MD, USA), with 130 "L- shaped" geometric figures. These geometric figures are homogeneously distributed between 10 rows and 13 columns. The different tissue structures (cardiomyotubules, non-fibrous connective tissue, or subtypes of collagen fibers) were distinguished and counted according to the coincident points between the tissue structure and the "L"-vertices. After the distinction and counting of all absolute points (vertices), these values were relativized, and the proportion (%) of these points was obtained. The distinction between different tissue components of the cardiac muscle was facilitated by Gomori reticulin staining. This method is selective and distinctive for type I and type III collagen fibers because type III collagen fibers are argyrophilic and showing black staining.[31] Additionally, type I collagen fibers stained brown. The combination of Gomori's reticulum-staining method with stereological analysis is effective for the quantification of the constituents of cardiac muscle tissue. In addition, the employment of 30 photomicrographic fields (at 400x magnification) allowed the analysis of an extensive tissue area of all animals in each experimental group. The stereological analysis was performed in the image analyzing system, in the Image Pro-Plus program for Windows (v 6.1; Media Cybernetics Inc., Silver Spring, MD, USA). All images were obtained using a Zeiss Scope A1 microscope under conventional light conditions.

Statistical analysis

Statistical analyses and graph confections were performed using GraphPad Prism (v. 9.0; GraphPad Software, Boston, Massachusetts, USA). All data are expressed as mean ± standard error of the mean. Cardiac function data were analyzed using one-way analysis of variance with the Newman Keuls post hoc test. Morphometric and stereological data were analyzed using an unpaired Student's t-test. Statistical significance was set at $p < 0.05$.

Results

Effects of sodium overload during postnatal phases on cardiac function

In the basal condition, there were no differences in the LVESP (control: 95.6 ± 11.7 vs. experimental: 96.9 ± 15.5 mmHg; Fig. 1a), LVEDP (control: 10.4 ± 0.7 vs. experimental: 12.0 ± 1.5 mmHg; Fig. 1b), +dP/dt (control: 2296 ± 291.2 vs. experimental: 2360 ± 398.7 mmHg/s; Fig. 1c), -dP/dt (control: 1630 ± 253.3 vs. experimental: 1551 ± 283.5 mmHg/s; Fig. 1d), HR (control: 233.8 ± 19.4 bpm vs. experimental: 246.8 ± 16.8 bpm; Fig. 1e), perfusion pressure (control: 89.7 ± 11.5 mmHg vs. experimental: 78.8 ± 12.3 mmHg; Fig. 1f) and LVDevP (control: 85.2 ± 11.8 mmHg vs. experimental: 84.9 ± 14.8 mmHg; Fig. 1g) between the control (n = 8) and experimental (n = 8) groups. Similarly, during the reperfusion period, there were no differences between the control and experimental groups in the LVESP (control: 86.1 ± 8.4 vs. experimental: 83.2 ± 9.2 mmHg; Fig. 1a), +dP/dt (control: 2033 ± 181.8 mmHg/s vs. experimental: 1607 ± 353.0 mmHg/s; Fig. 1c), -dP/dt (control: 1247 ± 161.1 mmHg/s vs. experimental: 924.6 ± 160.3 mmHg/s; Fig. 1d), HR (control: 238.4 ± 21.7 bpm vs. experimental: 235.5 ± 13.9 bpm; Fig. 1e), perfusion pressure (control: 75.1 ± 9.4 mmHg vs. experimental: 78.8 ± 9.0 mmHg; Fig. 1f) and LVDevP (control: 74.5 ± 8.7 mmHg vs. experimental: 60.1 ± 11.2 mmHg; Fig. 1g). However, the experimental group showed a significant increase in the LVEDP during the reperfusion period as compared to control group (control: 11.6 ± 1.4 mmHg vs. experimental: 23.1 ± 5.2 mmHg; Fig. 1b; $p < 0.05$).

Effects of sodium overload during postnatal phases on reperfusion arrhythmias

All animals in the control (n = 8) and experimental (n = 8) groups subjected to the reperfusion protocol presented with arrhythmias. The experimental group showed an increase in the duration of arrhythmias (control: 75.0 ± 7.8 s vs. experimental: 208.8 ± 32.9 s; Fig. 2a; $p < 0.05$) and ASI (control: 1.0 ± 0.4 vs. experimental: 3.0 ± 0.4; Fig. 2b; $p < 0.05$) in relation to control group.

Effects of sodium overload during postnatal phases on histological and morphometric analysis of heart

Figure 3 shows representative photomicrographs of left ventricle cross sections of the control (A) and experimental (B) groups stained with Gömori's reticulin. The morphometric analysis showed that the VMI was diminished in the experimental group (n = 8) compared to control group (n = 8) (control: 0.24 ± 0.01 g/cm vs. experimental: 0.2 ± 0.01 g/cm; Fig. 3c; $p < 0.05$). These data indicate that sodium overload during postnatal phases does not promote cardiac hypertrophy in adult animals.

Histological analysis demonstrated that the differences in the relative frequencies of non-fibrous connective tissue were significantly smaller in the experimental group (control: $2.5 \pm 0.5\%$ vs. $1.0 \pm 0.3\%$; Fig. 3d; $p < 0.05$). A significant increase was observed in the relative frequency of collagen type I in the hearts of experimental animals (control: $15.8 \pm 0.7\%$ vs. experimental: $19.8 \pm 1.3\%$; Fig. 3e; $p < 0.05$). However, no difference was observed in the relative frequency of collagen type III in the hearts of the experimental animals compared to that in the control group (control: $32.4 \pm 1.0\%$ vs. experimental group: $34.0 \pm 1.1\%$; Fig. 3f). There was no difference in LCSA of cardiomyocytes of the experimental (n = 200 cardiomyocytes) and control (n = 200 cardiomyocytes) groups (control: 14.7 ± 0.2 μm vs. experimental: 15.0 ± 0.2 μm;). There was no difference in the relative frequencies of the cardiomyocytes between the control (n = 30 cardiomyocytes) and experimental (n = 30 cardiomyocytes) groups (control: $48.3 \pm 1.7\%$ vs. experimental: $45.3 \pm 1.4\%$).

Discussion

Despite the impact of HSD on cardiac function has been extensively examined, the influence of a high-salt diet during infancy adolescence on cardiac function in adulthood remains to be investigated. Under basal conditions, we observed that sodium overload during postnatal phases did not change the cardiac function of isolated hearts from adult rats; these hearts presented an important impairment of diastolic function, in addition to an increase in the severity of arrhythmias after ischemia/reperfusion. This is the first report showing that HSD during postnatal phases potentially increases the chance of death caused by severe arrhythmia after an anoxia event, such as infarction and cardiac arrest, in adulthood.

The results obtained in the present study may be attributed to potential permanent alterations induced by sodium overload during the early life stages. A previous study utilizing the same diet employed in the present investigation reported permanent increases in ABP and HR, along with a decrease in baroreflex sensitivity and hypernatremia-induced renal vasodilation.[21] In this
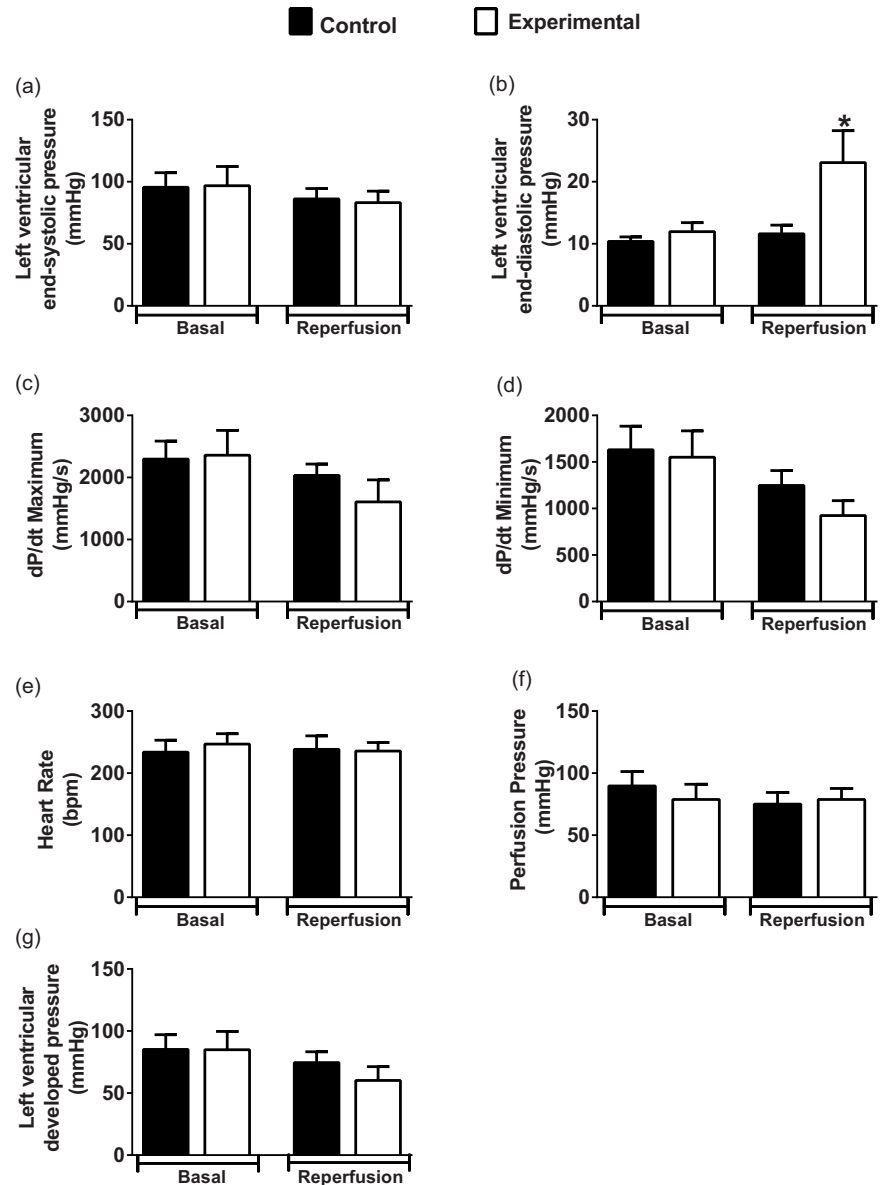
Figure 1. Effects of sodium overload during postnatal phases on isolated rat hearts. Values of left ventricular end-systolic pressure (***a***; mmHg), left ventricular end-diastolic pressure (***b***; mmHg), dP/dt maximum (***c***; mmHg/s), dP/dt minimum (***d***; mmHg/s), heart rate (***e***; bpm), perfusion pressure (***f***; mmHg), and left ventricular developed pressure (***g***; mmHg) in the control (black bar, $n = 8$) and experimental (white bar, $n = 8$) groups. Values are mean ± SEM. * $p < 0.05$, compared with the control group.
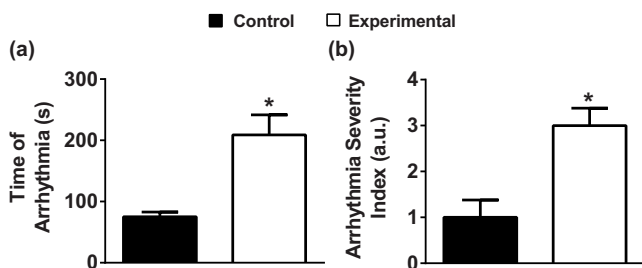

Figure 2. Effects of sodium overload during postnatal phases on reperfusion arrhythmias in isolated hearts. Duration of arrhythmia (***a***; s), arrhythmia severity index (***b***; a.u.) in the control (black bar; $n = 8$) and experimental (white bar; $n = 8$) groups. Values are mean ± SEM. * $p < 0.05$, compared with the control group.

way, the HSD can exert systemic effects and induce responses even after the withdrawal of the stimulus under free conditions.

The DOHaD hypothesis posits that environmental factors during early life, including the prenatal and postnatal periods, have profound impacts on health and disease outcomes in later life. In the present study, the salt overload protocol extended through the third cardiometabolic programing window, followed by a 30-day post-treatment observation period, thereby allowing outcomes to be attributed to the DOHAD phenomenon. The rationale for subjecting rats to high-salt diets during infancy–adolescence stems from the recognized plasticity and pivotal developmental significance inherent in this period. Infancy adolescence is characterized by heightened susceptibility to environmental stimuli and dynamic physiological changes, making it an opportune stage for investigating the enduring consequences of sodium overload on cardiovascular health, extending well into adulthood. In addition, evidence underscores the widespread consumption of processed and industrialized foods among contemporary children and adolescents, which leads to a high-sodium intake.[32,33] Given the established correlation between excessive sodium consumption and adverse cardiovascular outcomes,[18,19] it is imperative to explore the potential long-term ramifications of such dietary practices during critical periods of
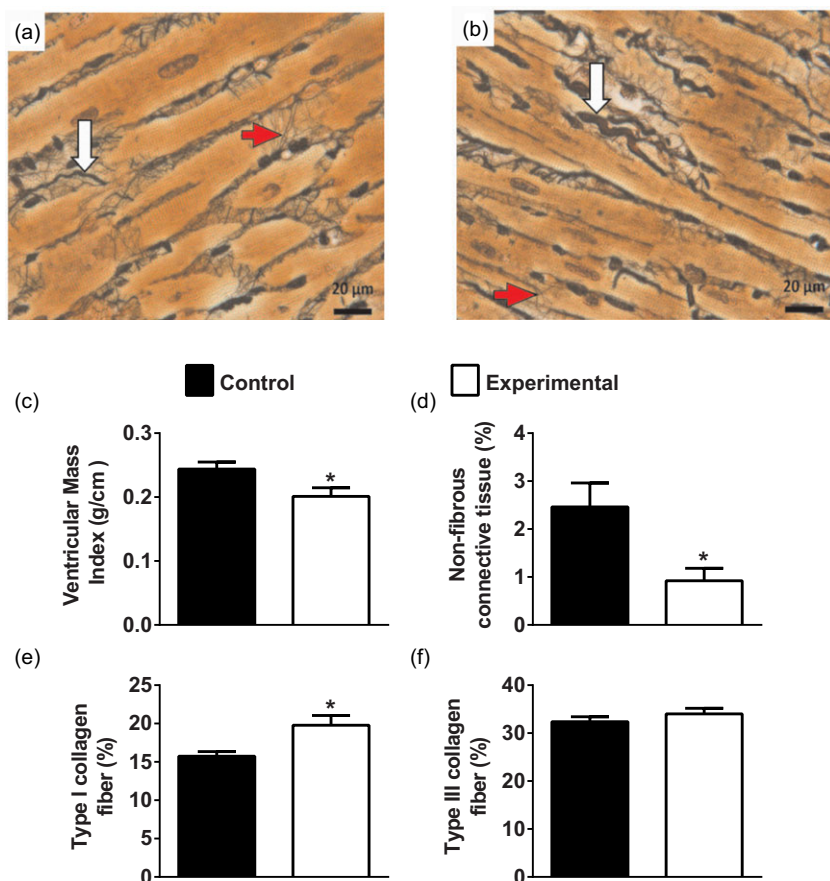
Figure 3. Representative photomicrographs (400x magnification) of the cross sections of the left ventricle of control animals (**a**) and animals subjected to sodium overload during postnatal phases (**b**) stained with Gömori reticulin. Type I collagen fibers are indicated by white arrows and type III collagen fibers are indicated by red arrows. Effects of sodium overload during postnatal phases on ventricular mass index (**c**; g/cm), non-fibrous connective tissue (**d**; %), collagen type I (**e**; %), and collagen type III (**f**; %) in the left ventricle of control (black bar; $n = 8$) and experimental (white bar; $n = 8$) groups. Values are mean ± SEM. * $p < 0.05$, compared with the control group.

growth and development. Taken together, the present study design not only aligns with the physiological plasticity characterizing this developmental phase, but also mirrors the modern dietary milieu experienced by youth, thereby augmenting the relevance and translational impact of our findings.

Several factors contribute to the development of diastolic disturbance,[34] such as progressive and sustained hypertensive state.[35] Some researchers have associated salt consumption with left ventricular diastolic dysfunction under hypertensive and normotensive conditions.[36,37] It has been suggested that the correlation between increased sodium consumption and diastolic dysfunction is linked to a mechanism of electrophysiological nature, in which the increases in intracellular Ca^{2+} concentration due to excessive salt intake were found to be underlying factors for myocardial relaxation compromise.[37] Our data extend these findings and demonstrate that HSD during infancy promotes diastolic dysfunction in hearts subjected to I/R.

In the present study, cardiomyocyte hypertrophy was not observed in the animals subjected to HSD. Thus, it is possible that diastolic dysfunction during reperfusion resulted from inadequate electrophysiological responses, possibly caused by the increased intracellular Ca^{2+} concentration or the dysfunction of Ca^{2+} reuptake during muscular relaxation. Interestingly, HSD increases the release of endogenous cardiac glycosides, which inhibit Na^+/K^+-ATPase.[38] This process can enhance ischemia-induced Na^+/K^+-ATPase inhibition, thereby blocking the Na^+/Ca^{2+} exchanger and resulting in a Ca^{2+} overload. Furthermore, oxidative stress contributes to ischemia-reperfusion mechanisms.[39] Under ischemia-reperfusion conditions, Ca^{2+} overload is associated with low

production of nitric oxide and high production of reactive oxygen species, contributing to reperfusion injuries,[40,41] such as arrhythmias and diastolic dysfunction. Oxidative stress also plays an important role in the modulation of the extracellular matrix, including an increase in collagen I.[9,42] In agreement with our data, it has been previously demonstrated that high-sodium intake increases oxidative stress resulting in myocardial injuries associated with extracellular matrix deposition.[9,43,44] Therefore, it is worth suggesting that high-sodium intake during postnatal phases promotes greater oxidative stress, which may lead to maladaptive left ventricle remodeling and consequent diastolic dysfunction. This proposition is supported by our findings of an increase in the relative frequency of collagen type I in adulthood in the hearts of animals subjected to sodium overload during infancy and adolescence.

In addition to an increase in intracellular Ca^{2+} concentration, diastolic dysfunction can result from cardiac tissue fibrosis. HSD can cause myocardial fibrosis in both hypertensive and normotensive experimental models, promoting left ventricular hypertrophy as well as intramyocardial fibrosis, which participates in abnormal myocardial stiffness and systolic and diastolic dysfunctions.[45] Different research groups have shown that myocardial fibrosis can be caused by a pressure overload or high-salt intake, separately.[45,46]

Studies have demonstrated that the long-term imbalances in the synthesis and degradation of collagen fibers from one type to another affect cardiac function.[47,48] In fact, investigations have demonstrated that an increase in the deposition of type I collagen fibers substantially enhances the cardiac ability to resist excessive

stretch.[48,49] Consistent with these findings, our study demonstrated an increase in type I collagen quantity in rats subjected to HSD, which may contribute to the diastolic dysfunction observed in these animals after an anoxia/reperfusion event.

The increase in collagen deposition in the heart tissue can compromise the electrical coupling between cardiomyocytes, thereby impairing the propagation of the electrical impulse and generating abnormal and desynchronized contraction of the cardiac muscle cells, characterizing arrhythmias.[6] HSD is a determining factor of arrhythmias in normotensive animals subjected to myocardial infarction because premature ventricular beats are increased in salt-loaded rats.[50] In fact, in our study, the hearts of animals subjected to sodium overload showed an increase in collagen type I deposition. Therefore, the increases in collagen deposition due to HSD, which we found, may increase the resistance to the propagation of the electrical impulse, serving as a mechanistic path and increasing the incidence and duration of reperfusion arrhythmias.

Conclusion

Taken together, our findings represent the first evidence that excessive sodium intake during the infancy-adolescence phases can impair cardiac function and promote arrhythmogenic effects after ischemia/reperfusion in adulthood. These experimental results suggest that sodium overload during the postnatal period can induce electrophysiological and morphological changes in the heart, potentially increasing the risk of death following reperfusion. However, further studies are required to elucidate the mechanisms underlying these responses.

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