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ORIGINAL CONTRIBUTION

Cardiac changes in apoptosis, inflammation, oxidative stress, and nitric oxide system induced by prenatal and postnatal zinc deficiency in male and female rats

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Abstract
Purpose Zinc restriction during fetal and postnatal development could program cardiovascular diseases in adulthood. The aim of this study was to determine the effects of zinc restriction during fetal life, lactation, and/or post-weaning growth on cardiac inflammation, apoptosis, oxidative stress, and nitric oxide system of male and female adult rats.
Methods Wistar rats were fed a low- or a control zinc diet during pregnancy and up to weaning. Afterward, offspring were fed either a low- or a control zinc diet until 81 days of life. IL-6 and TNF-α levels, TUNEL assay, TGF-β1 expression, thiobarbituric acid-reactive substances that determine lipoperoxidation damage, NADPH oxidase-dependent superoxide anion production, antioxidant and nitric oxide synthase activity, mRNA and protein expression of endothelial nitric oxide synthase, and serine1177 phosphorylation isoform were determined in left ventricle.
Results Zinc deficiency activated apoptotic and inflammatory processes and decreased TGF-β1 expression and nitric oxide synthase activity in cardiac tissue of both sexes. Male zinc-deficient rats showed no changes in endothelial nitric oxide synthase expression, but a lower serine1177 phosphorylation. Zinc deficiency induced an increase in antioxidant enzymes activity and no differences in lipoperoxidation products levels in males. Females were less sensitive to this deficiency exhibiting lower increase in apoptosis, lower decrease in expression of TGF-β1, and higher antioxidant and nitric oxide enzymes activities. A zinc-adequate diet during postnatal life reversed most of these mechanisms.
Conclusion Prenatal and postnatal zinc deficiency induces alterations in cardiac apoptotic, inflammatory, oxidative, and nitric oxide pathways that could predispose the onset of cardiovascular diseases in adult life.

Keywords Moderate zinc deficiency · Prenatal and postnatal growth · Cardiac apoptosis · Cardiac oxidative stress · Cardiac nitric oxide system · Sex differences

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Introduction

Many chronic cardiovascular diseases (CVDs) such as hypertension and heart failure have their origin during development [1, 2]. Fetal programming and sex differences in CVDs due to micronutrient deficiencies are an emerging area of investigation. Moderate zinc restriction during pregnancy could be a nutritional insult to fetal and postnatal development [3–5]. Zinc deficiency was originally thought to be of rare occurrence, but it is now estimated to affect more than 25% of the world's population [6]. According

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to a World Health Organization report (WHO 2002), zinc deficiency ranks fifth among the most important health risk factors in developing countries, and eleventh worldwide [7].

Zinc is an essential micronutrient involved in cardiac physiology due to the large number of zinc-dependent biological processes. It is important for cellular proliferation and the function of thousands of enzymes and transcription factors. Moreover, this micronutrient has antioxidant, anti-inflammatory, and anti-apoptotic properties [8, 9]. It has been reported to limit the extent of damage induced by free radicals, thereby suppressing some of the signaling pathways leading to apoptosis. Additionally, many studies describe that zinc directly inhibits certain apoptotic regulators, mainly caspase-3, caspase-6, caspase-9, and calcium–magnesium-dependent endonuclease [10–12]. Furthermore, several studies have shown that reduced circulating zinc concentration correlates with increased plasmatic levels of IL-6, IL-8, and TNF-α, leading to inflammation [13, 14]. Moreover, many enzymes that are involved in the regulation of the cardiovascular system, such as nitric oxide (NO) synthase (NOS), contain zinc in their structure. The NOS family consists of three isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS), which are expressed in many tissues, including vascular beds and the heart. These enzymes catalyze the synthesis of NO and L-citrulline from L-arginine in the presence of NADPH and O_2. All NOS isoforms contain a zinc thiolate (ZnS4) cluster that plays an essential role in the catalytic activity of this enzyme by maintaining stability of the dimer interface and integrity of the tetrahydrobiopterin binding site [8, 15, 16].

In previous studies, we showed that dietary zinc restriction during prenatal and postnatal growth induces an increase in arterial blood pressure and impairs renal function in adult male rats. These alterations were associated with an increase in renal oxidative stress and lower renal and vascular NOS activity [17–20].

In addition, we previously demonstrated that this nutritional injury alters cardiac development in males, evidenced by a reduction in myocytes size and in left ventricle (LV) wall thicknesses, as well as by a decreased contractility and dilatation of the LV in adult life. Moreover, zinc-deficient males showed a hypertrophic remodeling of the coronary artery architecture associated with this chronic increase in arterial blood pressure. These morphological alterations were not accompanied by interstitial fibrosis or collagen deposition, as expected in males that exhibited higher blood pressure levels. On the other hand, females would be less sensitive to this micronutrient deficiency since they exhibited normal levels of systolic blood pressure (SBP) and no significant structural or functional heart alterations [21].

In this regard, it has been shown that transforming growth factor beta (TGF-β) plays a crucial role in cardiac disease during adult life [22–24]. Besides, this growth factor is essential for epithelial–mesenchymal transformation during heart development [25–28]. There are three TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β. Of these, TGF-β1 is found almost ubiquitously and has been the most studied, since it is the main isoform involved in fibrotic diseases [29]. Moreover, this isoform is essential for proper development of the heart. Evidences have described that mice lacking TGFβ1 die in utero due to vascular defects and surviving mice develop inflammatory responses in different organ, including the heart [30].

Based on these previous studies, we hypothesized that moderate zinc restriction during development results in disturbances in NO system, oxidative stress, growth factors, apoptosis, and inflammatory processes in the cardiac tissue that can predispose the onset of CVDs in male and female adult rats. We further hypothesized that adequate zinc intake during postnatal life could not completely reverse the detrimental effects of earlier micronutrient imbalance on the cardiac tissue.

Therefore, to address this hypothesis, in the present study we evaluated the effects of zinc restriction during fetal life, lactation, and/or post-weaning growth on pro-inflammatory cytokines, oxidative stress, apoptosis, and the NO system in the heart of male and female adult rats. Moreover, we evaluated the importance of sex differences in the development of the different alterations observed in the cardiac tissue.

Materials and methods

Animals and study design

Female (f) Wistar rats weighing 280 ± 10 g obtained from the breeding laboratories of Facultad de Farmacia y Bioquímica (Universidad de Buenos Aires, Argentina) were mated by exposure to Wistar males (m) for 1 week. Animals were cared for according to Argentina's National Drug, Food and Medical Technology Administration Standards (Regulation 6344/96) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, Revised 1996). Experimental procedures were approved by the ethics committee for the care and use of laboratory animals of Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina (Res 3191).

Mothers and their offspring were housed in plastic cages in a humidity- and temperature-controlled environment with a 12-h light–dark cycle and were allowed food and deionized water ad libitum.

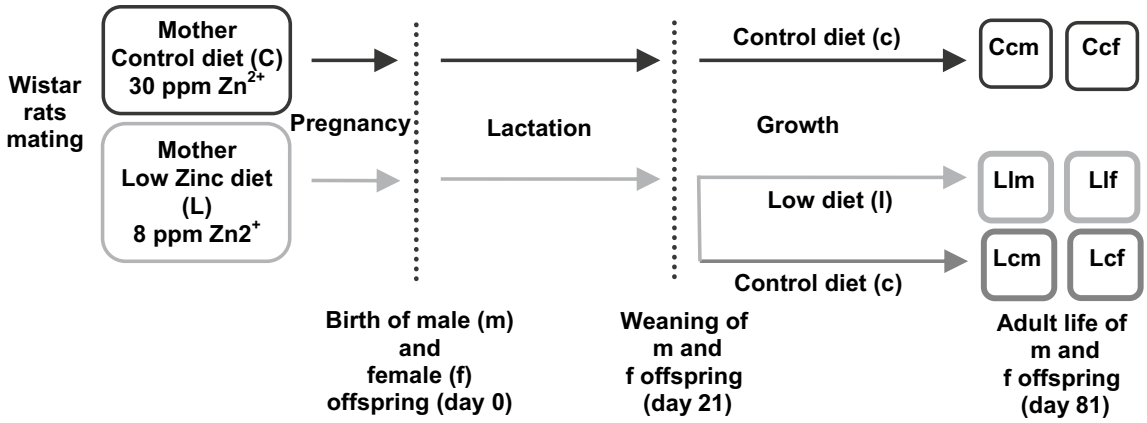

Fig. 1 Experimental animal model of moderate zinc deficiency during fetal life, lactation, and/or postnatal life

Female rats were randomly fed either a moderately zinc-deficient diet (L, 8 ppm, $n = 10$) or a control zinc diet (C, 30 ppm, $n = 5$) during the pregnancy and lactation periods. Eight rat pups remained with each mother until weaning (21 days of life) by random culling of pups at birth and retaining a 1:1 m-to-f ratio. After weaning, m and f offspring of L mothers were fed a low- (Llm and Llf, 8 ppm) or a control (Lcm and Lcf, 30 ppm) zinc diet for 60 days, and m and f offspring of C mothers were fed a control zinc diet (Ccm and Ccf, 30 ppm) (Fig. 1). Both diets contained all the necessary nutrients, except zinc content, to meet rat requirements for the pregnancy and lactation periods in accordance with AIN-93 recommendations [20, 31].

At 81 days of life, offspring of each diet group were killed by cervical decapitation. Immediately after obtaining a blood sample, the heart was excised and placed in an ice-cold saline solution, and then quickly dabbed for excess fluid prior to recording heart weight. In order to evaluate cardiac apoptosis and pro-inflammatory cytokines, the heart of a group of rats was fixed in 4% formaldehyde for 24 h and then transferred to 70% ethanol, trimmed, and embedded in paraffin. LV not destined to histochemical techniques, were separated, weighed, frozen in liquid nitrogen, and stored at −80 °C until analysis or placed in RNAlater RNA stabilization Reagent (QIAGEN, Limburg, the Netherlands) at −20 °C. Right tibia length (TL) was measured as a growth parameter.

Blood from offspring at 81 days of life and from mothers at weaning was collected to determine zinc concentration in plasma samples using atomic absorption spectrophotometry (Varian Spectrophotometer Spectr-AA-20, air-acetylene flame, 0.5 nm slit, wavelength of 213.9 nm) (PerkinElmer Corp, Norwalk, CT) [20].

Immunohistochemistry of pro-inflammatory cytokines and TUNEL assay

Paraffin-embedded sections of cardiac tissue were subjected to immunohistochemical assays using the commercial Vectastain ABC Kit (Universal Elite, Vector Laboratories, Burlingame, CA). Sections were deparaffinized with xylene and rehydrated through graded series of ethanol to water. Endogenous peroxidase was blocked in 1% w/v hydrogen peroxide in methanol for 30 min. Non-specific antibody binding was blocked with bovine serum for another 20 min. Sections were then incubated overnight at 4 °C with either goat polyclonal anti-IL-6 antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or rat monoclonal anti-TNF-alpha antibody (TNF-α; dilution 1:50; R&D Systems, Minneapolis, MN), diluted with blocking solution (PBS 1% bovine serum). Immunostaining was carried out with an avidin–biotin–peroxidase complex kit (using diaminobenzidine as chromogen) and counterstaining with hematoxylin. In all cases, two independent observers performed a blinded fashion evaluation. All results for histological staining were expressed as mean percentage of positive immunostaining/mm^2 from ten random images (magnification 400×) viewed and evaluated [32].

The DeadEnd Colorimetric TUNEL System, a non-radioactive kit designed to end-label the fragmented DNA of apoptotic cells, was used as previously described [18]. The number of TUNEL-positive cells per LV area was counted in 20 visual fields (magnification 400×) for each rat. Measurements were taken under similar light, gain and offset conditions by two blinded, independent observers.

Immunohistochemistry and TUNEL assays were analyzed using an Olympus BX51 light microscope equipped

with a digital camera (QColor 3, Olympus America, Inc., Richmond Hill, Ontario, Canada) connected to the Image-Pro Plus 4.5.1.29 software (Media Cybernetics, LP, Silver Spring, MD, USA).

Cardiac oxidative stress

Oxidative stress in LV was evaluated as previously described [33]. Lipid oxidative damage was assessed measuring the extent of formation of 2-thiobarbituric acid-reactive substances (TBARS; nmol/mg protein) [34]. Glutathione content was measured and expressed as mg/mg protein [35]. Superoxide dismutase (SOD) activity was assessed by measuring the ability of the homogenate to inhibit autoxidation of epinephrine and was expressed as units of SOD (U)/mg protein [36]. Catalase (CAT) activity was determined by the conversion of hydrogen peroxide to oxygen and water and was expressed as pmol/mg protein [37]. The assay described by Flohé and Gunzler [38] was used to measure glutathione peroxidase (GPx) activity, expressed as nmol/min.mg protein. Protein concentration was determined by the method of Bradford [39].

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent superoxide anion production in LV was measured using the lucigenin-enhanced method as described previously, with some modifications [40]. Briefly, LV slices (1 × 5 mm) were incubated in 3 ml of assay buffer during 20 min under 95% O_2–5% CO_2 aeration at 37 °C. The assay buffer consisted of Krebs buffer with the following composition (mM): 118.3 NaCl, 4.7 KCl, 1.35 $CaCl_2$, 1.2 $MgSO_4$, 1 K_2HPO_4, 25 $NaHCO_3$, 11 glucose, 20 Hepes (pH 7.4 after with 95% O_2–5% CO_2).

Each slice was placed in a scintillation vial containing 5 µmol/l lucigenin in a final volume of 1 ml warm Krebs-HEPES buffer (37 °C), and the emitted chemiluminescence (background) was measured using an LKB Wallac 1209 Rackbeta Liquid Scintillation Counter (Turku, Finland) in the chemiluminescence mode. Then, 5 mmol/l NADPH was added, and the emitted luminescence was measured for 5 min every 15 s. To assess the specificity of the method, LV slices were incubated with the SOD mimetic tempol (1 mM) in a final volume of 3 ml of assay buffer during 20 min under 95% O_2–5% CO_2 aeration at 37 °C and the measurements were taken in the presence of tempol (1 mM) in a final volume of 1 ml. Incubation of tempol reduced NADPH-stimulated luminescence signal to background levels in all groups.

Data were corrected for background activity, and the area under the curve (AUC) was calculated. The results were normalized to dry tissue weight, and the fold change was calculated versus Ccm values [41].

Cardiac NOS activity

NOS activity in LV was evaluated as previously described [42]. LV slices (2–3 mm thick) were incubated for 30 min at 37 °C in Krebs solution with [^{14}C]L-arginine 0.5 µCi/ml (specific activity 360 mCi/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA). To determine the activity of NOS isoforms, tissue slices were previously incubated with 1 mM aminoguanidine (AG; inducible NOS-specific inhibitor); 10 mM 7-nitroindazole (7-NI; neuronal NOS-specific inhibitor); 1 µM calmidazolium (Cz; calcium–calmodulin antagonist); 1 µM L-nitro-arginine-methyl-ester (L-NAME; NOS non-specific inhibitor) for 15 min. Tissue samples were homogenized in the stop solution (EGTA 0.5 mmol/L, EDTA 0.5 mmol/l, HEPES 20 mmol/l, pH 5.5), and the homogenates were centrifuged at 12,000*g* for 20 min. The supernatants were applied to a 1-ml Dowex AG 50 W-X8 column (Na^+ form; Bio-Rad laboratories, Hercules, CA), eluted with 2 ml of distilled water, and the amount of [^{14}C]L-citrulline was determined with a liquid scintillation counter (Wallac 1414 WinSpectral, EG&G Company, Turku, Finland). NOS activity was expressed as picomoles of [^{14}C]L-citrulline per gram of wet tissue per minute. All drugs were purchased from Sigma (St. Louis, MO).

Cardiac eNOS mRNA expression by RT-qPCR

RNA extraction and purification was performed using the RNeasy Fibrous Tissue Mini Kit (QIAGEN) after stabilization of the LV in RNAlater RNA Stabilization Reagent (QIAGEN). Stabilized tissue (15–20 mg) was added to the extraction buffer and then disrupted and homogenized using a rotor–stator homogenizer (Pellet Pestle Motor, Kontes, Rockwood, TN). Extraction and purification was completed with an on-column DNase treatment (RNase-Free DNase Set, QIAGEN) in accordance with the protocol described by the kit manufacturer.

RNA quantity and quality were analyzed using a BioTek Synergy HT (BioTek Instruments, Winooski, VT). RNA extractions showed a 260/280 ratio of approximately 1.9–2.0, confirming good RNA quality. The RNA samples were stored at −80 °C.

cDNA was synthesized from 1 µg of total RNA using RevertAid Reverse Transcriptase with $Oligo(dT)_{18}$ primers, dNTP Mix, Ribolock™ RNase Inhibitor (Thermo Scientific, Waltham, MA). cDNA samples were stored at −20 °C.

Real-time PCR analysis was performed in a 72-well rotor on a Rotor-Gene Q instrument (QIAGEN) using the double-stranded DNA-specific fluorochrome SYBR Green.

Table 1 Primer sequence

Gene	Sequence (5′–3′)	Product size (bp)
eNOS	Fw: GCAAGACCGATTACACGACA Rv: GTCCTCAGGAGGTCTTGCAC	207
GAPDH	Fw: CCTGCACCACCAACTGCTTAGC Rv: GCCAGTGAGCTTCCCGTTCAGC	239

eNOS endothelial NOS, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase

All the primers used in this study were designed using the Primer 3 free software and were purchased from IDT (San Diego, CA). All these primers amplified only one fragment of the expected size. Amplification efficiency was determined by the dilution method using a cDNA pool and an initial dilution of 1/10 for eNOS and GAPDH, calculated using the Rotor-Gene Q analysis software, QIAGEN. The list of primers used, characteristics of the amplicons, and the resulting efficiencies are shown in Table 1.

Reactions were performed in a volume of 25 µl containing 6 µl cDNA, 0.5 µM forward and reverse primers, and 12.5 µl SYBR Green Master Mix 2x (Biodynamics, Argentina). Each sample was analyzed twice, and a non-template control for each primer set was included in the real-time plates. Amplification conditions were as follows: 94 °C for 2 min; 40 cycles of 94 °C for 15 s, 58 °C for 35 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplification specificity was verified by subjecting the products to melting curve analysis where temperature was increased from 77 to 95 °C, and fluorescence was monitored.

Threshold values were obtained using the automated setting of the instrument software which considers fluorescence when the maximum PCR efficiency is reached. The Cq (quantification cycle) value was automatically calculated by the software. Data, expressed as Cq values, were imported into the curve to obtain the copies per reaction for each sample. eNOS mRNA results were normalized to GAPDH expression, and the fold change was calculated versus Ccm values.

Cardiac eNOS, p-eNOS (Ser 1177), and TGF-β_1 protein expression by Western blot analysis

LV was homogenized and centrifuged. Equal amounts of protein (50 µg protein/lane) were separated by electrophoresis in 7.5% SDS–polyacrylamide gels, transferred to a nitrocellulose membrane (Amersham G.E. Healthcare, United Kingdom), and incubated with rabbit polyclonal anti-eNOS (BD Transduction Laboratories), anti-pSer1177-eNOS (Cell Signaling, Danvers, MA), anti-TGF-β_1 (ABCAM, Cambridge, United Kingdom) and anti-β-actin (Sigma-Aldrich, San Luis, MO) antibodies diluted 1/750, 1/1000, 1/750, and 1/2000 in 1% BSA, respectively, and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted 1/3000 (Bio-Rad, Hercules, CA).

Samples were detected by chemiluminescence using ECL Prime Western Blotting Detection Reagent (Amersham, GE Healthcare). Relative protein expression was quantified from the autoradiograph using a scanner (HP Photosmart C3180) and ImageJ software (National Institutes of Health). Optical density (OD) of eNOS was normalized to β-actin OD, pSer1177 eNOS OD was normalized to eNOS OD, and the fold change was calculated versus Ccm values. All experiments were performed in triplicate.

Statistical analysis

All values are expressed as mean ± SEM. The Prism program (Graph Pad Prism 5.0 Software, Inc., San Diego, CA) was used for statistical analysis. The mean and standard error values of each experimental group were analyzed with a two-way analysis of variance (ANOVA). One factor was diet and the other was sex (male or female). The effects of one factor were tested independently of the effects of the other. If diet × sex interaction was not significant, two-way ANOVA was performed followed by a Bonferroni post hoc test for multiple comparisons of main effects. When diet × sex interaction was significant, the simple effects were analyzed by a Bonferroni post hoc test for multiple comparisons between groups of interest. p value <0.05 was considered a significant difference.

Results

Offspring body and heart weight

At 81 days of life, Llm, Llf, Lcm, and Lcf offspring showed lower body weight, TL, heart weigh/TL and LV weight/TL values than Ccm and Ccf, respectively. Furthermore, these growth markers were lower in female than in male groups (Table 2).

Zinc concentration in plasma

At weaning, L mothers showed lower plasmatic zinc concentration than C ones [L (n = 10): 38 ± 6 µg/dl vs. C (n = 5): 76 ± 6 µg/dl, $p < 0.0001$]. Llm and Llf rats showed lower zinc concentration in plasma than Ccm, Ccf, Lcm, and Lcf rats at 81 days of life (Ccm: 160 ± 9; Llm: 120 ± 5*§; Lcm: 147 ± 10; Ccf: 158 ± 6; Llf: 106 ± 7$^{\dagger a}$; Lcf: 150 ± 6 µg/d; *$p < 0.01$ vs. Ccm; $^{\dagger}p < 0.01$ vs. Ccf, $^{\S}p < 0.05$ vs. Lcm, $^{a}p < 0.05$ vs. Lcf, n = 18 per group). There were no sex differences in plasmatic zinc concentration.

Table 2 Body weight, tibia length, and cardiac weight at 81 days of life

	Ccm	Llm	Lcm	Ccf	Llf	Lcf
BW (g)	407 ± 4	321 ± 10*	345 ± 10*	264 ± 3*	224 ± 2†,‡	229 ± 5†,§
TL (cm)	4.00 ± 0.03	3.72 ± 0.03*	3.76 ± 0.02*	3.60 ± 0.02*	3.47 ± 0.02†,‡	3.41 ± 0.02†,§
CW/TL (g/cm)	0.30 ± 0.01	0.26 ± 0.01*	0.26 ± 0.01*	0.24 ± 0.01*	0.20 ± 0.01^{†,‡,a}	0.22 ± 0.01†,§
LV/TL (g/cm)	0.204 ± 0.004	0.189 ± 0.003*	0.193 ± 0.003*	0.168 ± 0.001*	0.143 ± 0.002^{†,‡,a}	0.153 ± 0.003†,§

BW body weight, *TL*: tibia length, *CW* cardiac weight, *LV* left ventricle. Control male (Ccm), Control female (Ccf), Low low male (Llm), Low low female (Llf), Low control male (Lcm), and Low control female (Lcf) zinc diet groups. Values are mean ± SEM, $n = 12$ per group

LV, LV/TL, CW/TL: two-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons of main effects. Diet and sex factors were considered significant: * $p < 0.05$ versus Ccm; † $p < 0.05$ versus Ccf, ‡ $p < 0.05$ versus Llm; § $p < 0.05$ versus Lcm; ^{a} $p < 0.05$ versus Lcf. Sex × diet interaction: not significant

BW, TL: two-way ANOVA, sex × diet interaction: significant ($p < 0.01$). Simple effects were analyzed by a Bonferroni post hoc test for multiple comparisons between groups of interest: * $p < 0.05$ versus Ccm; † $p < 0.05$ versus Ccf, ‡ $p < 0.05$ versus Llm; § $p < 0.05$ versus Lcm

Table 3 Pro-inflammatory cytokines levels and apoptotic cells in left ventricle at 81 days of life

	Ccm	Llm	Lcm	Ccf	Llf	Lcf
TNF-α (% of positive staining/mm^2)	1.4 ± 0.4	20.4 ± 2.0*,§	1.7 ± 0.6	1.4 ± 0.3	21.2 ± 1.9^{†,a}	1.7 ± 0.3
IL-6 (% of positive staining/mm^2)	1.8 ± 0.3	21.1 ± 1.7*,§	2.8 ± 0.8	1.7 ± 0.4	20.8 ± 1.6^{†,a}	2.5 ± 0.7
TUNEL (No. of apoptotic cells/area)	5 ± 2	44 ± 2*,§	4 ± 3	5 ± 2	20 ± 2^{†,‡,a}	6 ± 3

Control male (Ccm), Low low male (Llm), Low control male (Lcm), Control female (Ccf), Low low female (Llf), Low control female (Lcf) zinc diet groups. Values are mean ± SEM, $n = 6$ per group. Data were analyzed using two-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons of main effects. Diet and sex factors were considered significant: * $p < 0.05$ versus Ccm; † $p < 0.05$ versus Ccf; ‡ $p < 0.05$ versus Llm; § $p < 0.05$ versus Lcm; ^{a} $p < 0.05$ versus Lcf. Sex × diet interaction: not significant

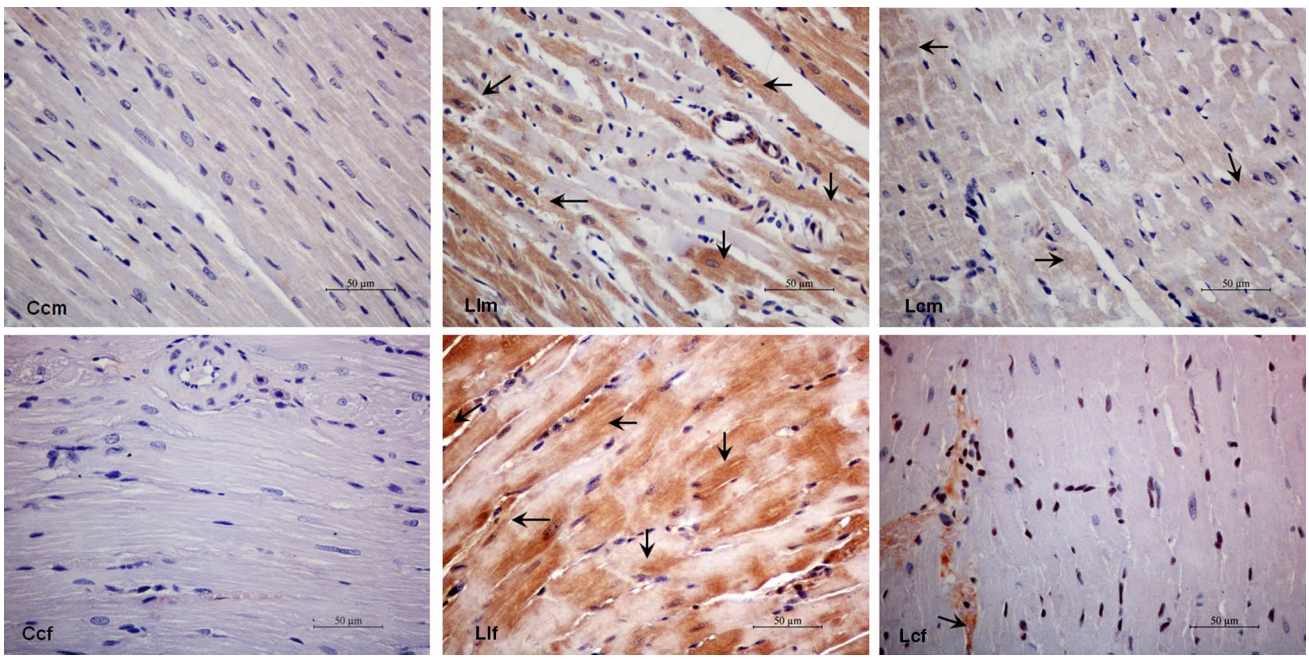

Fig. 2 Immunohistochemistry of TNF-α in LV. Control male (Ccm), Low low male (Llm), Low control male (Lcm), Control female (Ccf), Low low female (Llf), Low control female (Lcf) at 81 days of life. All images are at the same magnification (×400). *Scale bar* 50 µm

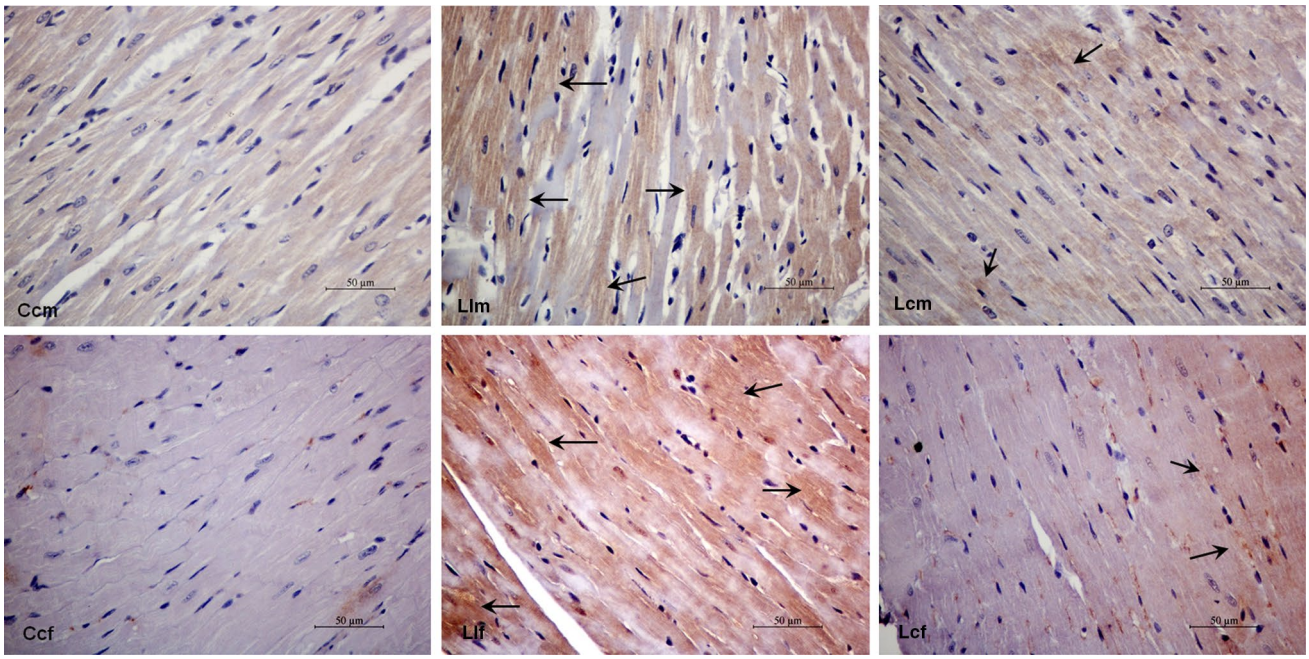

Fig. 3 Immunohistochemistry of IL-6 in LV. Control male (Ccm), Low low male (Llm), Low control male (Lcm), Control female (Ccf), Low low female (Llf), Low control female (Lcf) at 81 days of life. All images are at the same magnification (×400). *Scale bar* 50 µm

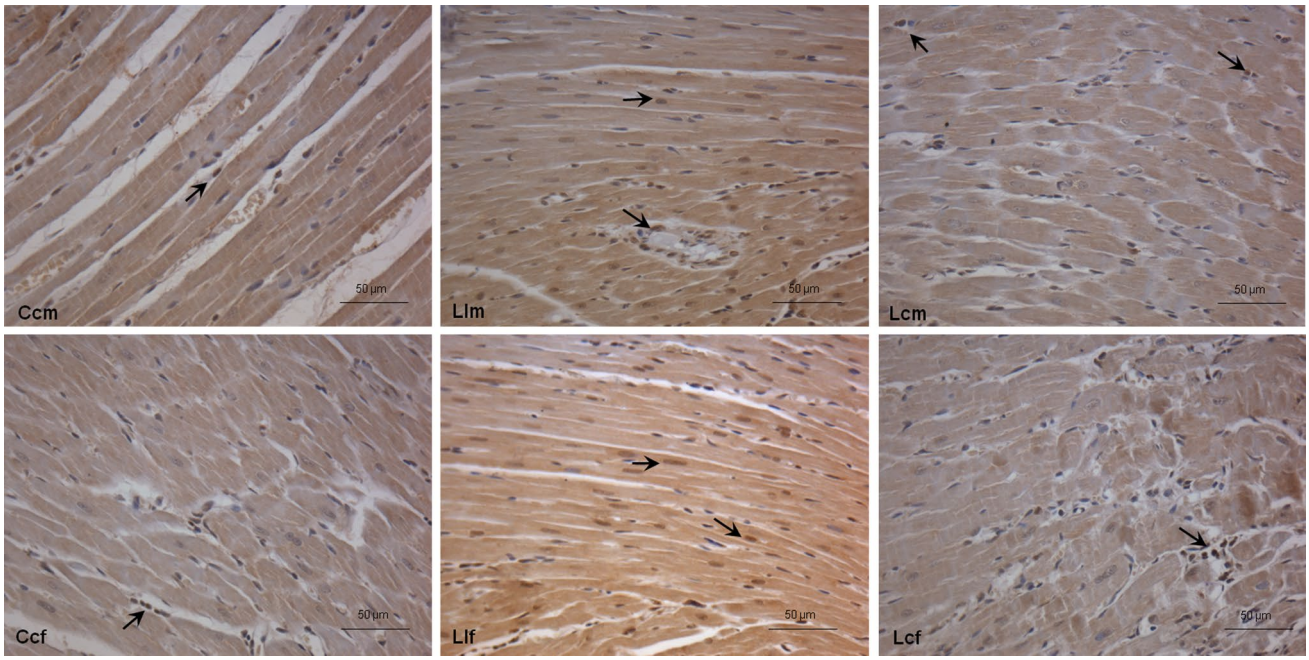

Fig. 4 Histochemistry of TUNEL assay in LV. Control male (Ccm), Low low male (Llm), Low control male (Lcm), Control female (Ccf), Low low female (Llf), Low control female (Lcf) at 81 days of life. All images are at the same magnification (×400). *Scale bar* 50 µm

Immunohistochemistry of pro-inflammatory cytokines, TUNEL assay, and TGF-β_1 protein expression in left ventricle

At 81 days of life, Llm and Llf rats exhibited higher levels of IL-6 and TNF-α than Ccm, Lcm, Ccf, and Lcf, respectively. There were no sex differences in IL-6 and TNF-α immunostained area in LV (Table 3; Figs. 2, 3).

Examination of TUNEL-stained LV sections at the end of the experimental period revealed an increased number

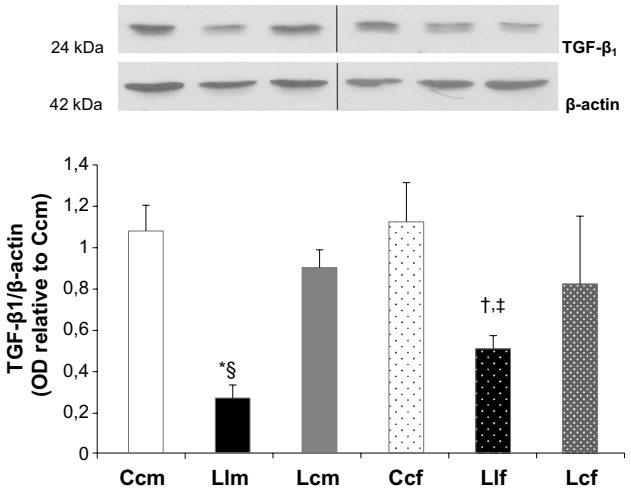

Fig. 5 TGF-β_1 protein expression determined in LV at the end of the experimental protocol in Control male (Ccm), Control female (Ccf), Low low male (Llm), Low low female (Llf), Low control male (Lcm) and Low control female (Lcf) zinc diet groups. Values are mean ± SEM, $n = 6$ per group. Data were analyzed using two-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons considering main effects. Diet and sex factors were considered significant: $^{*}p < 0.05$ versus Ccm; $^{\dagger}p < 0.05$ versus Ccf; $^{\ddagger}p < 0.05$ versus Llm; $^{\S}p < 0.05$ versus Lcm. Sex × diet interaction: not significant. *White spaces* in the representative blots of TGF-β_1 and β-actin demarcate non-contiguous gel lanes from the same gel

of apoptotic cells in Llm and Llf rats compared with Ccm, Lcm, Ccf, and Lcf, respectively. However, Llf showed a lower increase in the number of apoptotic cells than LLm (Table 3; Fig. 4).

Cardiac TGF-β_1 protein content was lower in Llm and Llf rats than in Ccm, Lcm, Ccf, and Lcf rats, respectively. TGF-β_1 protein expression in LV was similar in Ccm and Ccf offspring, but the decrease in expression of this growth factor was lower in zinc-deficient females than in males (Fig. 5).

Cardiac oxidative stress

NADPH oxidase-dependent superoxide anion production, glutathione concentration, and TBARS levels, as well as SOD, CAT, and GPx activities, were measured in LV tissue at the end of the experimental period (Table 4). NADPH oxidase-dependent superoxide anion production was significantly higher in Llm group compared to Ccm and Lcm groups. Concerning antioxidant enzymes, males exposed to moderate zinc deficiency (Llm and Lcm) exhibited higher GPx activity than Ccm. Moreover, Lcm exhibited higher SOD activity than Ccm, but there were no significant differences in catalase activity among these groups.

Females showed lower NADPH oxidase-dependent superoxide anion production and higher activity of antioxidant enzymes than males. There were no differences in glutathione concentration among the experimental groups. Moreover, lipid oxidative damage measured by the concentration of TBARS in the cardiac tissue was similar in all the experimental groups.

Cardiac NO system

Figure 6 shows NOS activity measured in LV at 81 days of life. Llm, Llf, Lcm, and Lcf offspring showed lower basal NOS activity than Ccm and Ccf, respectively. Ccf, Llf, and Lcf exhibited higher basal NOS activity levels than Ccm,

Table 4 Antioxidant and oxidant systems in left ventricle at 81 days of life

	Ccm	Llm	Lcm	Ccf	Llf	Lcf
NADPH oxidase-dependent superoxide anion production (AUC/mg dry weight)	1.00 ± 0.01	1.68 ± 0.09*,§	0.81 ± 0.10*	0.51 ± 0.07*	0.64 ± 0.06‡	0.77 ± 0.06
TBARS (nmol/mg protein)	0.084 ± 0.006	0.083 ± 0.018	0.077 ± 0.010	0.112 ± 0.024	0.097 ± 0.012	0.092 ± 0.015
SOD (U/mg protein)	3.36 ± 0.33	3.78 ± 0.20§	6.41 ± 0.39*	5.31 ± 0.46*	5.87 ± 0.55‡	4.16 ± 0.49§
CAT (pmol/mg protein)	0.052 ± 0.003	0.063 ± 0.005	0.044 ± 0.005	0.072 ± 0.008*	0.077 ± 0.006‡	0.058 ± 0.004§
GPx (pmol/min/mg protein)	80.3 ± 3.0	117.0 ± 5.9*	106.8 ± 3.7*	108.5 ± 8.5*	106.3 ± 8.5	96.3 ± 10
GLUT (mg/mg protein)	0.064 ± 0.005	0.059 ± 0.007	0.066 ± 0.009	0.070 ± 0.004	0.060 ± 0.004	0.062 ± 0.005

AUC area under the curve, *GLUT* glutathione concentration, *TBARS* 2-thiobarbituric acid-reactive substances concentration, *SOD* superoxide dismutase activity, *CAT* catalase activity, *GPx* glutathione peroxidase activity. Control male (Ccm), Control female (Ccf), Low low male (Llm), Low low female (Llf), Low control male (Lcm), and Low control female (Lcf) zinc diet groups. Values are mean ± SEM, $n = 6$ per group

GLUT, TBARS, CAT: two-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons of main effects. Diet and sex factors were considered significant: $^{*}p < 0.05$ versus Ccm; $^{\ddagger}p < 0.05$ versus Llm; $^{\S}p < 0.05$ versus Lcm. Sex × diet interaction: not significant

NADPH oxidase-dependent superoxide anion production, GPx, SOD: two-way ANOVA, sex × diet interaction: significant ($p < 0.01$). Simple effects were analyzed by a Bonferroni post hoc test for multiple comparisons between groups of interest: $^{*}p < 0.05$ versus Ccm; $^{\ddagger}p < 0.05$ versus Llm; $^{\S}p < 0.05$ versus Lcm

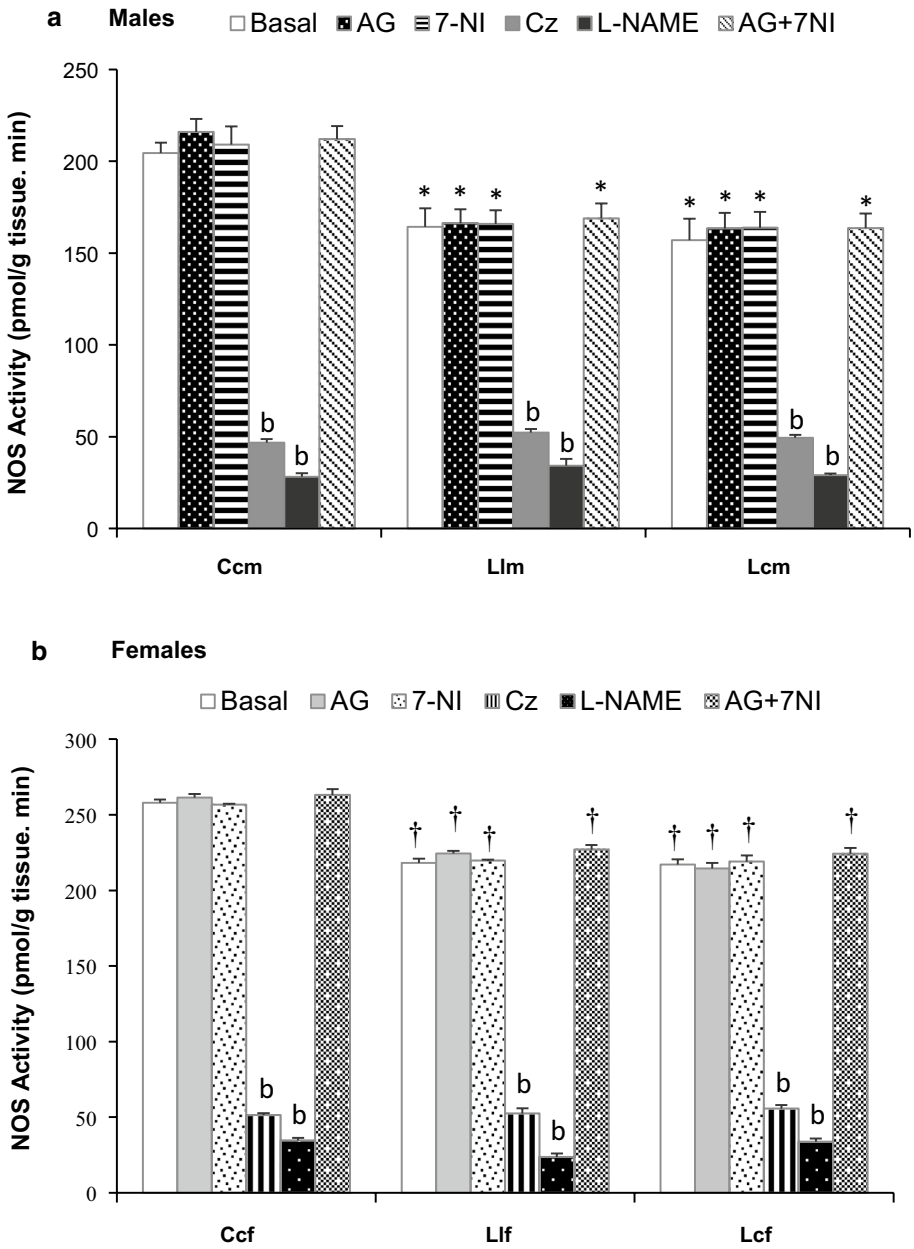

Fig. 6 NOS activity in LV at day 81 of life in **a** male and **b** female offspring. Zinc diet groups: Control male (Ccm), Control female (Ccf), Low low male (Llm), Low low female (Llf), Low control male (Lcm), and Low control female (Lcf). To determine the activity of NOS isoforms, tissue slices were previously incubated with 1 mM aminoguanidine (AG; inducible NOS-specific inhibitor); 10 mM 7-nitroindazole (7-NI; neuronal NOS-specific inhibitor); 1 µM calmidazolium (Cz; calcium–calmodulin antagonist); 1 µM L-nitro-arginine-methyl-ester (L-NAME; NOS non-specific inhibitor) for 15 min. Values are mean ± SEM, $n = 6$ per group. Data were analyzed using two-way ANOVA, sex × diet interaction: significant ($p < 0.0001$). Simple effects were analyzed by a Bonferroni post hoc test for multiple comparisons between groups of interest: $^{*}p < 0.05$ versus Ccm; $^{\dagger}p < 0.05$ versus Ccf, $^{b}p < 0.05$ versus basal

Llm, and Lcm, respectively (% increase in NOS activity vs. males: Ccf: 26%; Llf: 32%; Lcf: 38%).

Basal NOS activity was blunted when L-NAME was previously added, thus verifying that the activity measured was linked specifically to NOS. Basal NOS activity in zinc-deficient groups was abolished by using a calcium–calmodulin antagonist but not by using aminoguanidine (nNOS inhibitor) or 7-nitroindazole (iNOS inhibitor) (Fig. 6).

Given the effects of zinc deficiency on eNOS activity, we evaluated mRNA and protein expression of this NOS isoform. Moreover, we determined protein expression of eNOS phosphorylated at Ser1177 since it is one of the most studied posttranslational modifications that activate this enzyme [43]. As shown in Fig. 7a, there were no significant differences in eNOS mRNA expression among the studied groups.

Consistently with basal NOS activity, Ccf exhibited higher eNOS protein expression than Ccm. Moreover, pSer1177-eNOS protein abundance in cardiac tissue of Llf and Lcf was higher than in males. Zinc deficiency during fetal and postnatal life did not modify eNOS protein abundance in males, but Llm showed lower eNOS phosphorylation at Ser1177 than Ccm. Even though Lcm showed lower cardiac NOS activity, eNOS protein expression and its phosphorylation levels at Ser1177 were not affected. In females, Lcf exhibited lower eNOS protein expression accompanied by higher eNOS Ser1177 phosphorylation than Ccf (Fig. 7b, c).

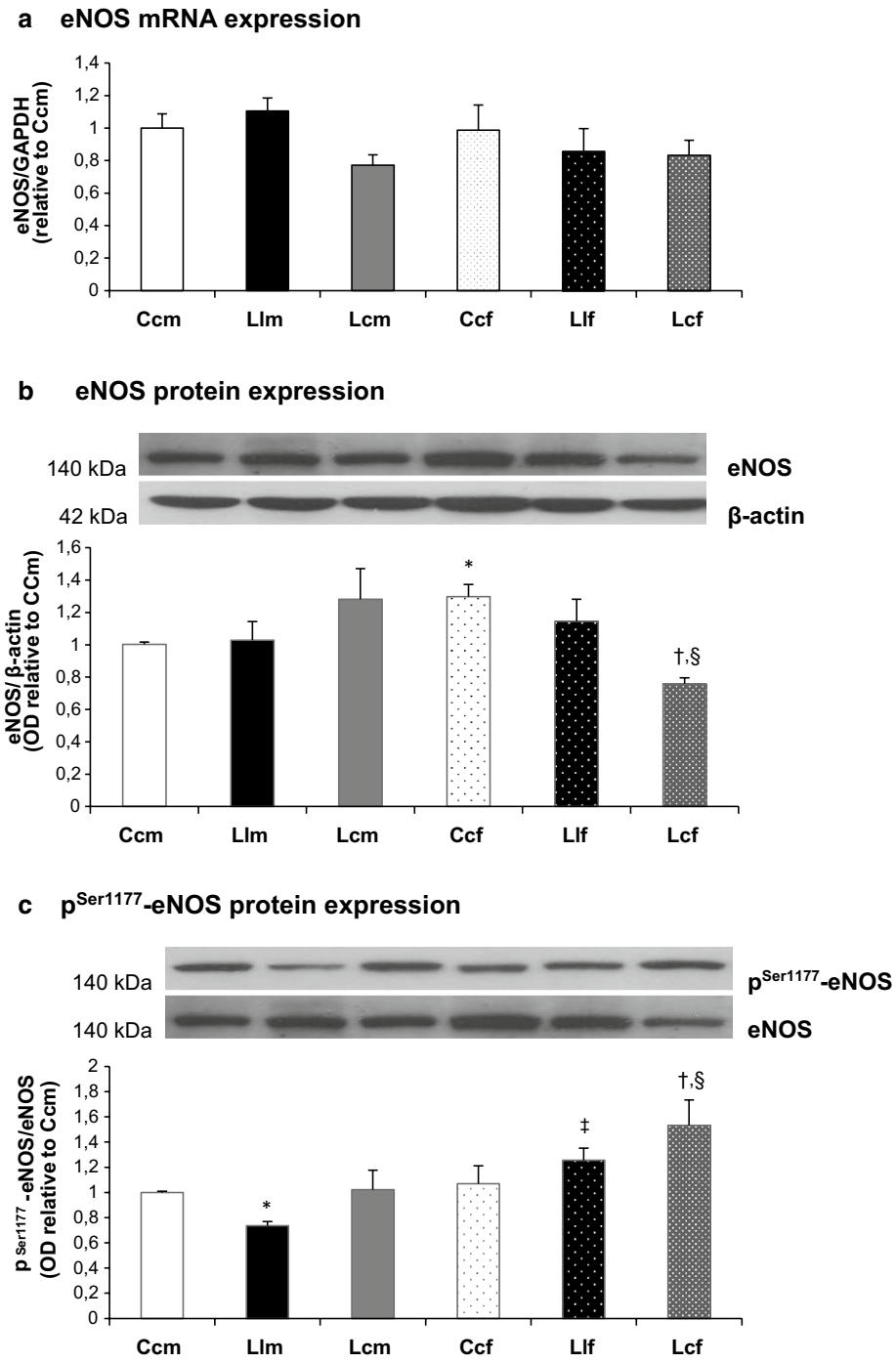

Fig. 7 **a** eNOS mRNA expression, **b** eNOS protein expression, and **c** pSer1177-eNOS protein expression determined in LV at the end of the experimental protocol in Control male (Ccm), Control female (Ccf), Low low male (Llm), Low low female (Llf), Low control male (Lcm), and Low control female (Lcf) zinc diet groups. Values are mean ± SEM, $n = 6$ per group. Data were analyzed using two-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons of main effects. eNOS mRNA expression: Diet and sex factors were considered not significant. Sex × diet interaction: not significant. eNOS and pSer1177-eNOS proteins expression: Diet and sex factors were considered significant: $^{*}p < 0.05$ versus Ccm; $^{\dagger}p < 0.05$ versus Ccf, $^{\ddagger}p < 0.05$ versus Llm; $^{\S}p < 0.05$ versus Lcm. Sex × diet interaction: not significant

Discussion

The results of the present study demonstrate that moderate zinc deficiency during critical periods of prenatal and postnatal development induces alterations in biological zinc-dependent cardiac processes such as apoptosis, inflammation, oxidative stress, and NO system that could contribute to the onset of the morphological and functional cardiac alterations previously described in this animal model [21]. Moreover, as in many developmental programming models, we found that male and female offspring exhibit different phenotypes following insults in utero, as well as differences in the severity of CVDs [44, 45].

In the present study, we demonstrate that moderate zinc restriction during fetal life, lactation, and postnatal life increases cardiac apoptosis and pro-inflammatory

cytokine levels in male adult rats. Moreover, sustained zinc deficiency during prenatal and postnatal growth determines lower protein expression of TGF-β_1 in adult hearts. Together, these cellular and molecular alterations could contribute, at least in part, to program the cardiac growth delay evidenced by diminished heart and LV weights. These data correlate with our earlier studies that showed decreased myocytes diameters, smaller LV wall thicknesses, larger LV diameter, and impaired LV contractile function in adult Llm rats [21]. Moreover, we previously showed that Llm and Lcm offspring developed an increase in SBP at 81 days that was not accompanied by compensatory cardiac hypertrophy and fibrosis [21]. Probably, an inadequate balance between apoptosis and the expression of growth factors, like TGF-β_1, could also explain, in part, these previous findings. This hypothesis is supported by diverse studies reporting that apoptosis is an integral component in cardiac development and zinc deficiency can induce a disruption in patterns of apoptosis and proliferation. It has been reported that maternal zinc deficiency is associated with excessive embryogenic cell death, especially in highly populated regions of neural crest cells [46]. Moreover, TGF-β_1 is a growth factor involved in heart development and cardiac remodeling in adulthood. During development, TGF-β_1 is involved in cell growth, migration, adhesion, survival, extracellular matrix formation, cell proliferation, and cell differentiation [24–26], and during adult life, it has been recognized to play a critical role in cardiac disease. When TGF-β_1 levels increase, as in ischemic disease, hypertension, heart failure, and valvular disease, there is a higher fibroblast proliferation rate, deposition of extracellular matrix constituents, and hypertrophy of cardiac myocytes [27–29]. However, further investigation is needed to determine how zinc deficiency can induce downregulation in TGF-β_1 expression in cardiac tissue, as well as the possible association between lower TGF-β_1 levels and decreased cardiac cell signaling and function.

On the other hand, female seemed to be less influenced by this nutritional injury during prenatal and postnatal life. Even though this micronutrient deficiency induced growth delay in female rats, the effects in the expression of TGF-β1 and in the number of apoptotic cells in LV were lower in Llf than in Llm. The present results are in agreement with our previous study that evidence that these animals do not show significant echocardiographic heart alterations [21]. Taking these evidences into account, it would be interesting to investigate the possible mechanisms involved in these sex differences. It has been demonstrated that physiological concentrations of 17β-estradiol have anti-apoptotic properties in cultured cardiac myocytes [47]. In addition, it has been described that the expression and activity of certain growth factors, such as TGF-β, are sex-dependent and depend on the presence of steroid hormones [48, 49].

Moreover, post-weaning restitution of a zinc-adequate diet restored the apoptotic and pro-inflammatory parameters, as well as the expression levels of TGF-β1, to Ccm and Ccf values. These results are consistent with other studies where zinc chelation in cell culture medium causes apoptosis and subsequent addition of zinc protects cells against the undergoing apoptosis, even if it is added to the cell culture only a short time after an apoptotic agent [50]. Moreover, in experimental human zinc deficiency model, it was shown that even a mild deficiency increased the production of pro-inflammatory cytokines and zinc supplementation could potentially have a beneficial effect [12, 13].

Nevertheless, the restitution of a zinc-adequate diet during post-weaning life could not normalize growth markers in adult males. These results highlight the importance of this micronutrient during fetal life and lactation in cardiac development. Since regulation of apoptosis and proliferation during the first week of life is essential for appropriate heart development, we hypothesize that increased cell apoptosis and/or decreased proliferation during this stage of growth would program morphological alterations that could not be overcome by a control zinc diet in later period of life. This hypothesis would be partially supported by our previous results showing an increased cardiac apoptosis at 6 days of life in zinc-deficient rats [21].

On the other hand, zinc acts as an antioxidant inducing the generation of metallothioneins, proteins rich in cysteine, and binding to the sulfhydryl (SH) groups of biomolecules thus protecting them from oxidation. It also increases the activation of antioxidant systems and decreases the activity of oxidant-promoting enzymes, such as NADPH oxidase, and inhibits lipid peroxidation production [11, 12, 51].

The heart handles a large amount of reactive oxygen species (ROS) production since it is a tissue with high-energy requirements. This characteristic of cardiac tissue determines a well-developed antioxidant capacity [52]. The heart handles a large amount of reactive oxygen species (ROS) production since it is a tissue with high-energy requirements. This characteristic of cardiac tissue determines a well-developed antioxidant capacity [52]. In the present study, we showed that moderate zinc deficiency determines higher levels of GPx in Llm and Lcm. The higher activity of this enzyme would contribute to decreased hydrogen peroxide production in LV and consequently the production of ROS involved in lipid oxidation [53]. Moreover, SOD activity was increased only after post-weaning restitution of a zinc-adequate diet in males. Considering that SOD is a zinc-dependent enzyme, zinc deficiency could not allow its activation in Llm. In addition, the higher production of superoxide anion induced by NADPH would evidence a higher capacity of oxidant-promoting enzymes in Llm. These results lead us to hypothesize that there is an alteration in oxidative stress capable of

activating antioxidant defenses in Llm and Lcm, allowing them to maintain levels of ROS involved in cardiac lipid peroxidation. Since the distribution of zinc and antioxidant capacity varies greatly among different tissues/organs, the impact of zinc deficiency on oxidative stress is clearly tissue-dependent. In this regard, we have demonstrated that moderate zinc deficiency during fetal and postnatal growth induces a different oxidative stress situation in renal tissue, characterized by higher lipid peroxidation levels and lower antioxidant enzyme activities and glutathione levels [19].

Moreover, our results show that Ccf rats exhibited lower NADPH oxidase-dependent superoxide anion production and greater SOD, CAT, and GPx activity in LV than Ccm rats. This characteristic determines a protected status in female offspring exposed to zinc deficiency, allowing them to better neutralize a possible excess in ROS production. Consistent with our findings, male offspring from a maternal low-protein diet model presented increased protein carbonyl concentrations and glutathione peroxidase activity in the liver while these programmed changes were absent in the female offspring [54]. Although the causes of this sexual dimorphism have not been elucidated, previous studies have suggested that estrogen upregulates SOD expression and activity in a time and dose manner [55].

Inflammation, apoptosis, and oxidative stress are closely interrelated pathways that can be modulated by zinc [56]. Previous studies described that zinc can inhibit the activation of apoptosis and pro-inflammatory cytokines expression in myocytes since it controls the activation of transcription factors involved in their signaling pathways, but also because it has an antioxidant activity [12, 57–59]. However, future studies are needed to elucidate how moderate zinc deficiency can affect the interrelated molecular signaling pathways among apoptosis, inflammation, and oxidative stress in the cardiac tissue of this animal model.

NO plays integral roles in the cardiovascular system, not only in the regulation of vascular smooth muscle tone but also in the function of ion channels, myocyte contraction, O_2 consumption, apoptosis, and myocardial remodeling [60–62]. As in renal and vascular tissues, we found lower LV NOS activity in Llm, Llf, Lcm, and Lcf offspring, associated mainly with lower eNOS activity [17–19]. The eNOS isoform is constitutively expressed in endothelial cells and myocytes in the LV. Since abundance of the eNOS isoform in the heart is higher in endothelium than in myocytes, it has been proposed that physiological eNOS signaling in vivo has paracrine functions. In fact, after being synthesized by endothelial cells, NO could diffuse and modify the biology of the neighboring cells such as cardiomyocytes [63]. Since in this study the NO system was measured in the whole LV and not in isolated myocytes, it was not possible to identify the source of NO production that is diminished.

This isoform has an important role in the maintenance of cardiac function. Several studies have evidenced that basal production of NO from eNOS and nNOS stimulates cardiac contractility by inducing a positive ionotropic effect and, further, it was demonstrated that NO increases oxygen consumption efficiency in myocardium and has positive chronotropic and lusitropic effect [61, 62]. Additionally, decreased eNOS expression levels were associated with higher myocardial and endocardial apoptosis [16] On the basis of this evidence and our previous study, we propose that a decrease production in NO could contribute, in part, to the lower ejection fraction observed in Llm. However, this would not be the main mechanism, since Lcm, Llf, and Lcf rats showed similar changes in NO system but exhibited no functional changes [21].

NOS has a cluster zinc thiolate in the dimer interface that is important for the activity of the enzyme [15]. We therefore hypothesized that zinc deficiency could alter the structure of this cluster leading to the lower activity found in these groups. Moreover, the redox environment, within the site of NO production, greatly influences NO bioavailability. A higher production of superoxide anion could reduce NO bioavailability by generating peroxynitrates [63]. However, in our experimental model, the compensated oxidative state induced by activation in antioxidant capacity would prevent an even lower availability of NO in cardiac tissue.

No alteration in eNOS protein and mRNA expression was found in Llm offspring, although decreased activation of eNOS regulated by Ser1177 phosphorylation could be contributing to lower LV NO production. Restitution of a zinc-adequate diet after weaning could not restore NOS activity in males, even though eNOS protein and mRNA expression and the levels of Ser1177 eNOS phosphorylation were similar to Ccm. Therefore, we consider that the decrease cardiac NOS activity in zinc-deficient rats could be related to different post-translational regulatory mechanisms, including alterations in (1) the dimeric enzyme structure; (2) the subcellular localization; (3) interactions with caveolin and heat–shock protein 90 (Hsp90); (4) availability of substrate (L-arginine) and cofactors (flavins, NADPH, and tetrahydrobiopterin (BH4); (5) other phosphorylation reactions; and (6) dephosphorylation, S-nitrosylation, and acetylation reactions [43, 64, 65]. Therefore, we postulate that disturbances in zinc homeostasis during fetal and lactation periods could affect some of these post-translational regulatory mechanisms leading to a reduced NOS activity that persists until adulthood. Moreover, the mechanisms altered by zinc deficiency during post-weaning life could be different to those affected in prenatal life.

On the other hand, Ccf showed higher NO system activity than Ccm since they exhibited higher NOS activity and eNOS protein expression in LV. Moreover, Llf and Lcf

showed a lower decrease in NOS activity compared with Llm and Lcm. Further, zinc-deficient females presented a compensatory activation of eNOS by increase in Ser 1177 phosphorylation that is more evident in Lcf since this group exhibited decreased eNOS protein expression. As in males, the alterations in the NO system were not associated with eNOS transcriptional changes. The data collected suggest a higher protected status in females, and this is consistent with other studies showing that treatment with 17β-estradiol increases the activity of calcium-dependent NO synthase (eNOS) in guinea pig heart [66].

In conclusion, the results of the present study provide strong evidence that zinc deficiency during prenatal and postnatal life induces alterations in apoptosis, inflammatory cytokines, growth factor expressions, oxidative stress state, and NO system in the cardiac tissue of male and female adult rats. These disturbances could contribute, in part, to the changes in heart structure and function previously described [21]. This nutritional injury induced a greater cardiac damage in males than in females, evidenced by increased apoptosis, activation of antioxidant capacity, decreased expression of TGF-β_1, and decreased NO system activity. Moreover, adequate zinc content in the diet during postnatal life could reverse most of these mechanisms, contributing to restoration of normal contractibility.

Therefore, the data strengthen the importance of diet optimization, not only during prenatal development to prevent programming of CVDs but also during postnatal life to overcome or diminish the cardiovascular consequences of this micronutrient deficiency during fetal life. Moreover, our experiments provide a baseline for future studies exploring the contribution of sexual hormones on the differences observed between males and females in this animal model.

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Author contributions MNG, FMGA, MED, GP, and LVJ involved in animal care and diet preparation. JET studied the immunohistochemistry of pro-inflammatory cytokines. FMGA, MED, and LVJ were responsible for the determination of cardiac oxidative stress. RE, MNG, FMGA, and LVJ assessed the cardiac NOS activity. MNG, ALT, and LVJ involved in cardiac eNOS mRNA expression by RT-qPCR. LVJ, GP, and AC studied cardiac eNOS, p-eNOS (Ser 1177), and TGF-β1 protein expression by Western blot analysis. LVJ, OP, and LG performed TUNEL assay. MNG, FMGA, LVJ, and ALT involved in NADPH oxidase-dependent superoxide anion production. CTA, RE, and ALT were responsible for experimental design, scientific and technical supervision, and analysis of results. LVJ, ALT, RE, and CTA drafted the manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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