

## CALL FOR PAPERS | *Sex and Gender Differences in Cardiovascular Physiology - Back to the Basics*

# Morphological and functional effects on cardiac tissue induced by moderate zinc deficiency during prenatal and postnatal life in male and female rats

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**Tomat AL, Juriol LV, Gobetto MN, Veiras LC, Abregú FM, Zilberman J, Fasoli H, Elesgaray R, Costa MA, Arranz CT.** Morphological and functional effects on cardiac tissue induced by moderate zinc deficiency during prenatal and postnatal life in male and female rats. *Am J Physiol Heart Circ Physiol* 305: H1574–H1583, 2013. First published September 27, 2013; doi:10.1152/ajpheart.00578.2013.—The aim of this study was to evaluate whether moderate zinc restriction in rats throughout fetal life, lactation, and/or postweaning growth results in early changes in cardiac morphology predisposing the onset of cardiac dysfunction in adult life as well as sex-related differences in the adaptation to this nutritional injury. Female Wistar rats received low or control zinc diets from the beginning of pregnancy up to offspring weaning. After being weaned, offspring were fed either a low or control zinc diet until 81 days. Systolic blood pressure was measured. Echocardiographic and electrocardiographic examinations, morphological experiments, and apoptosis by TUNEL assay were performed in the left ventricle. In the early stages, zinc-deficient male and female offspring showed an increase in cardiomyocyte diameter, probably associated with an increase in cardiac apoptotic cells, but smaller myocyte diameters in adulthood. In adult males, this nutritional injury induced decreased contractility and dilatation of the left ventricle, not allowing the heart to compensate the higher levels of blood pressure, and hypertrophic remodeling of coronary arteries associated with increased blood pressure. Adequate zinc intake during postweaning life did not overcome blood pressure levels but reversed some of the detrimental effects of earlier zinc deficiency in cardiac morphology and function. Females were less sensitive to this deficiency, exhibiting normal levels of blood pressure and no structural or functional heart alterations in adult life. The present study demonstrates that the effects of zinc deficiency on blood pressure, cardiac morphology, and function differ between sexes, with males more predisposed to develop cardiovascular diseases in adulthood.

moderate zinc deficiency; prenatal and postnatal growth; heart morphology and function; sex differences

CARDIOVASCULAR DISEASE (CVD) is the major cause of morbidity and mortality in modern societies (19). CVD in humans develops over decades, but according to the theory of fetal programming, its cornerstone is already laid in childhood. The role of

micronutrients during prenatal and postnatal growth as dietary factors capable of programming CVD in adult life is an emerging area of investigation. The consequences of micronutrient imbalance on the developing fetus may not be apparent at the time of the nutritional insult but may manifest later in development (13, 32).

Moderate and marginal zinc deficiency observed in pregnant women could be a nutritional insult to fetal and postnatal development (37, 46). The Food and Agriculture Organization of the United Nations has estimated the prevalence of inadequate zinc intake to be as high as 20.5% worldwide (49). Zinc deficiency is usually due to inadequate zinc intake or absorption, increased losses of zinc from the body, or increased zinc requirements (5, 14, 23).

In previous studies, we demonstrated that moderate zinc deficiency in male rats during intrauterine and postnatal growth programs CVD and renal diseases in adult life. Dietary zinc restriction during fetal life and lactation induces an increase in arterial blood pressure and impairs renal function in adult male rats (40, 42, 43).

Zinc is an essential micronutrient with a potential link to cardiac physiology for its catalytic, regulatory, and structural functions in thousands of enzymes. In addition, 10% of genes encode zinc-containing proteins (22, 39). Moreover, this micronutrient is involved in the reduction of oxidative stress and in the inhibition of apoptosis and inflammation (45, 51).

Several studies have shown decreased blood zinc levels in adult patients with ischemia/myocardial infarction, dilated cardiomyopathy, congestive heart failure, conduction abnormalities, arrhythmias and coronary diseases, and arterial hypertension (1, 4, 8, 12, 17, 19, 31, 38). Furthermore, in a rodent model of ischemia-reperfusion, administration of zinc during reperfusion improves myocardial recovery and decreases arrhythmias (16).

On the other hand, heart development can be particularly sensitive to zinc deficiency, and it is an important target of fetal programming of CVD in adult life. In rats, severe maternal zinc deficiency has been associated with a high incidence of fetal heart anomalies (11, 20). Moreover, excessive embryonic cell death after maternal dietary zinc deficiency has been reported

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to occur in regions that are essential to support normal heart morphogenesis (20). However, much remains to be elucidated about the critical role of zinc ions during fetal and postnatal growth and cardiac pathophysiology.

During fetal development, myocardial growth is regulated by the controlled proliferation and apoptosis of cardiomyocytes (33). In the rat heart, cardiomyocytes continue to proliferate in the early neonatal period and commence the maturation process at around postnatal *day 3* or *4*, when they cease proliferation and become differentiated (18). Hence, the majority of postnatal cardiac growth is due to hypertrophy of existing cardiomyocytes and extracellular matrix deposition (33).

Therefore, in the present study, we hypothesized that moderate zinc restriction in male and female rats throughout fetal life, lactation, and/or postweaning growth results in early changes in cardiac morphology that can predispose the onset of cardiac dysfunction in adult life. 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. Finally, we evaluated the existence of sex differences in adaptations to this nutritional injury. To address this hypothesis, the objective of this study was to evaluate cardiac morphology and/or function in male and female 6-, 21-, and 81-day old rats exposed to zinc deficiency during fetal life, lactation, and/or postnatal growth.

## MATERIALS AND METHODS

**Animals and study design.** Female Wistar rats weighting  $280 \pm 10$  g obtained from the breeding laboratories of the Facultad de Farmacia y Bioquímica (Universidad de Buenos Aires, Buenos Aires, Argentina) were mated by exposure to Wistar males for 1 wk. Immediately afterward, female rats were randomly fed either a moderately zinc-deficient diet (L group, 8 ppm,  $n = 10$ ) or a control zinc diet (C group, 30 ppm,  $n = 8$ ) during the pregnancy and lactation periods. Eight rat pups remained with each mother until 6 or 21 days of life (weaning) by random culling of pups at birth and retaining a 1:1 male-to-female ratio. Experimental groups at 6 and 21 days were as follows: male offspring of C mothers (Cm group,  $n = 10$ ), female offspring of C mothers (Cf group,  $n = 10$ ), male offspring of L mothers (Lm group,  $n = 10$ ), and female offspring of L mothers (Lf group,  $n = 10$ ). After being weaned, male and female offspring of L mothers were fed a low-zinc diet (Ll group, 8 ppm,  $n = 10$ ) or control zinc diet (Lc group, 30 ppm,  $n = 10$ ) for 60 days, and male and female offspring of C mothers were fed a control zinc diet (Cc group, 30 ppm,  $n = 10$ ).

Both diets had all the necessary nutrients, except zinc content, to meet rat requirements for pregnancy and lactation periods according to AIN-93 recommendations (29, 42).

Animals were cared for according to Argentina's National Drug, Food and Medical Technology Administration Standards (Regulation 6344/96) and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). Experimental procedures were approved by the Ethics Committee of the School of Pharmacy and Biochemistry of the Universidad de Buenos Aires.

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

Offspring were weighed at 6, 21, and 81 days of life, and body weight gain was calculated. Systolic blood pressure (SBP) was measured indirectly in awake animals by the tail-cuff method (AD Instruments PowerLab 8/30 and Lab Chart 6 Pro software) at 36, 51, 66, and 81 days of life. Before SBP was measured, rats were warmed in a thermostated (30°C), quiet room for 40 min. The blood pressure

value for each rat was calculated as the average of six separate measurements. The first value measured was not used in the determination of SBP levels.

Echocardiographic and electrocardiographic examinations were performed in rats at 81 days of life. Animals were anesthetized with ketamine (100 g/kg ip) and midazolam (5 g/kg ip), and their ventral thorax and upper abdomen were shaved under aseptic conditions. Echocardiographic measurements were performed in the left lateral decubitus position. Two-dimensional directed M-mode images were obtained using a SonoScape (A6 Vet) system with a 4- to 9-MHz transducer. Measurements were taken from the right parasternal short-axis plane at the level of the mitral valve leaflets. Left ventricular (LV) internal diameter (LVID), LV posterior wall thickness (PWT), and anterior wall thickness (AWT) were measured at both systole (s) and diastole (d). Ejection fraction (EF) and fractional shortening (FS) were calculated from ventricular internal diameters. Relative LV wall thickness (RWT) was estimated using the following formula:  $RWT = [(PWTd + AWTd)/LVIDd]$ . All determinations were made according to the guidelines of the American Society of Echocardiography (35).

Cardiac rhythm was monitored using a standard ECG (model TM 1210, Temis). ECGs were recorded in the supine position using one electrode on each limb. The electrode on the right hindlimb functioned as a reference electrode. In accordance with American Heart Association recommendations, bipolar leads I (between the right and left forelimbs), II (between the right forelimb and left hindlimb), and III (between the left anterior limb and left posterior limb) and unipolar leads avR, AvL, and avF were obtained. ECG signals were recorded at a sampling rate of 25–50 mm/s. Registrations were performed with a minimum duration of 1 min to allow for beat selection and subsequent averaging.

At 6, 21, and 81 days of life, offspring in each diet group were euthanized by cervical decapitation. Immediately after a blood sample was drawn, the heart was excised, placed in ice-cold saline, and then quickly dabbed for excess fluid before heart weight was recorded. To evaluate cardiac morphology, the heart was fixed in 4% paraformaldehyde for 24 h and then transferred to 70% ethanol, trimmed, and embedded in paraffin.

Blood from offspring at 6, 21, and 81 days of life and from mothers at weaning was collected to determine serum zinc concentration using atomic absorption spectrophotometry (Varian Spectrophotometer Spectr AA-20, air acetylene flame, 0.5-nm slit, wavelength of 213.9 nm, Perkin-Elmer, Norwalk, CT) (41, 44).

**Histological evaluation of the LV at 6, 21, and 81 days.** The major and minor diameters of LV cardiomyocytes were measured in 4- $\mu$ m-thick cross-sections of the heart stained with hematoxylin and eosin at  $\times 400$  magnification. Transversely sectioned myocytes with central and nearly round-shaped nuclei from multiple regions of the LV were analyzed in 2 slides/rat (i.e., 100 cells/rat). Afterward, major and minor diameters were averaged to obtain mean cardiomyocyte diameter (MCD).

Heart sections were subjected to collagen-specific staining with picrosirius red to determine interstitial collagen fraction (ICF), perivascular collagen deposition (PVC), and coronary artery wall area (CWA) of the LV. ICF was expressed as a percentage of the total cardiac tissue area. PVC was expressed as the ratio of the collagen areas immediately surrounding the vessels to the vessel lumen areas (LA). CWA was calculated by subtracting LA from the corresponding total vessel area (TVA; excluding the adventitia) and expressed as a percentage of LA. The ratio of LA to TVA was calculated and expressed as a percentage.

The DeadEnd Colorimetric TUNEL system, a nonradioactive kit designed to end label the fragmented DNA of apoptotic cells, was used as previously described (19, 27). The number of TUNEL-positive cells per LV area was counted in 20 visual fields (magnification:  $\times 400$ ) for each rat in early postnatal life (6 and 21 days of life).

Table 1. *Body weight, heart weight, and serum zinc concentration at 6 and 21 of life*

	Cm Group	Lm Group	Cf Group	Lf Group
Body weight, g				
6 days	12.6 ± 0.6	10.2 ± 0.3*	12.3 ± 0.6	10.0 ± 0.3†
21 days	45 ± 2	34 ± 2*	45 ± 3	32 ± 2†
Heart weight/body weight, g/100 g				
6 days	0.68 ± 0.01	0.59 ± 0.01*	0.70 ± 0.02	0.61 ± 0.01†
21 days	0.62 ± 0.02	0.54 ± 0.01*	0.63 ± 0.03	0.56 ± 0.01†
Serum zinc concentration, µg/dl				
6 days	173 ± 5	103 ± 7*	177 ± 8	100 ± 10†
21 days	139 ± 9	95 ± 8*	131 ± 7	89 ± 9†

Values are means ± SE; *n* = 10 animals/group. The following groups are shown: male offspring of control diet-fed mothers (Cm group), male offspring of low-zinc diet-fed mothers (Lm group), female offspring of control diet-fed mothers (Cf group), and female offspring of low-zinc diet-fed mothers (Lf group). The factor of diet was considered significant: \**P* < 0.01 vs. the Cm group and †*P* < 0.01 vs. the Cf group; interaction sex × diet: not significant.

Histological and TUNEL assays were analyzed using an Olympus BX51 light microscope equipped with a digital camera (Qcolor 3 Olympus America) and connected to Image-Pro Plus 4.5.1.29 software. Measurements were performed blindly and under similar light, gain, and offset conditions by the same researcher.

**Statistical analysis.** All values are expressed as means ± SE. The Prism program (Graph Pad Software, San Diego, CA) was used for statistical analysis. Means and SEs of each variable were calculated for each group, and the results of each variable for each experimental group were analyzed with two-way ANOVA. The factors tested were diet versus sex (male or female) and diet versus time (6, 21, or 81 days). The effects of one factor were tested independently of the effects of the other, and no interaction between diet and sex or between diet and time was found. Multiple comparisons were performed using a Bonferroni post hoc test. Serum zinc concentration of mothers was analyzed by a Student's *t*-test. *P* values of <0.05 were considered significant.

## RESULTS

**Body weight gain and heart and body weights of offspring.** Lm and Lf offspring exhibited lower body and heart weights at 6 and 21 days compared with C offspring, with no differences between males and females (Table 1). Moreover, body weight gain between 6 and 21 days was lower in Lm and Lf rats than in control rats [Cm group: 33 ± 2 g, Lm group: 24 ± 2 g (diet factor: *P* < 0.01 vs. the Cm group), Cf group: 33 ± 2 g, and Lf group: 22 ± 2 g (diet factor: *P* < 0.01 vs. the Cf group), *n* = 10 rats/group].

At 81 days of life, male and female Ll and Lc rats showed lower body weights than Ccm and Ccf rats, respectively, whereas Llm and Lcm offspring showed lower heart weight/tibial length than Ccm offspring. There were no differences in heart weights among the female dietary groups (Table 2). Furthermore, male and female Ll and Lc groups exhibited lower body weight gain between 21 and 81 days than Ccm and Ccf groups, respectively

[Ccm group: 385 ± 8 g, Llm group: 325 ± 9 g (diet factor: *P* < 0.01 vs. the Ccm group), Lcm group: 358 ± 23 g (diet factor: *P* < 0.01 vs. the Ccm group), Ccf group: 234 ± 5 g (diet factor: *P* < 0.01 vs. the Ccm group), Llf group: 194 ± 5 g (diet factor: *P* < 0.01 vs. the Ccf group), and Lcf group: 206 ± 12 g (diet factor: *P* < 0.01 vs. the Ccf group), *n* = 10 rats/group]. These growth markers were lower in the Ccf group than in the Ccm group.

**Zinc concentration in plasma.** At weaning, L mothers showed lower serum zinc concentration than C mothers (L group: 35 ± 7 µg/dl vs. C group: 73 ± 7 µg/dl, *P* < 0.0001, *n* = 8–10 rats/group), whereas Lm and Lf offspring exhibited lower serum zinc concentrations than Cm and Cf offspring at 6 and 21 days of life (Table 1). Ll animals showed lower zinc concentrations in plasma than Cc and Lc rats of both sexes at 81 days of life (Table 2). There were no sex differences in zinc concentration in plasma.

**Offspring SBP.** Figure 1, *A* and *B*, show SBP values for male and female Cc, Lc, and Ll rats. Llm rats exhibited higher levels of SBP at 51, 66, and 81 days of life than Ccm rats, whereas Lcm rats showed higher SBP values at 66 and 81 days of life than Ccm rats. At 81 days, SBP values of Llm and Lcm animals were higher than 140 mmHg. However, there were no differences in SBP among Llf, Lcf, and Ccf animals at any experimental time. Moreover, control male and female offspring showed similar SBP levels.

**Offspring LV morphology and function.** Table 3 shows echocardiographic and heart rate data for all groups. In systole, Llm and Lcm rats had thinner AWTs and PWTs and larger LVIDs than Ccm rats. Llm rats had larger LVIDd and thinner AWTd and PWTd than Ccm rats. Furthermore, only in diastole, Lcm showed similar LVIDd and PWRd than Ccm. In addition, Llm and Lcm rats showed lower RWT than Ccm rats. Moreover, EF and FS, indirect measures of LV contractile function, were decreased in Llm compared with Ccm and Lcm, while there were no differences in echocardiographic param-

Table 2. *Body weight, heart weight, and serum zinc concentration at 81 days of life*

	Ccm Group	Llm Group	Lcm Group	Ccf Group	Llf Group	Lcf Group
Body weight (g)	431 ± 8	359 ± 8*	392 ± 23*	279 ± 5*	226 ± 7†	242 ± 14†
Heart Weight/tibial length (g/cm)	0.32 ± 0.02	0.24 ± 0.01*	0.22 ± 0.02*	0.22 ± 0.01*	0.20 ± 0.01	0.19 ± 0.01
Serum zinc concentration (µg/dl)	156 ± 10	117 ± 5*‡	143 ± 10	152 ± 6	107 ± 8†§	148 ± 6

Values are means ± SE; *n* = 10 animals/group. The following groups are shown: control diet-fed male offspring of control diet-fed mothers (Ccm group), low zinc diet-fed male offspring of low-zinc diet-fed mothers (Llm group), control diet-fed male offspring of low-zinc diet-fed mothers (Lcm group), control diet-fed female offspring of control diet-fed mothers (Ccf group), low-zinc diet-fed female offspring of low-zinc diet-fed mothers (Llf group), and control diet-fed female offspring of low-zinc diet-fed mothers (Lcf group). Factors of sex and diet were considered significant: \**P* < 0.01 vs. the Ccm group, †*P* < 0.01 vs. the Ccf group, ‡*P* < 0.05 vs. the Lcm group, and §*P* < 0.05 vs. the Lcf group; interaction sex × diet: not significant.

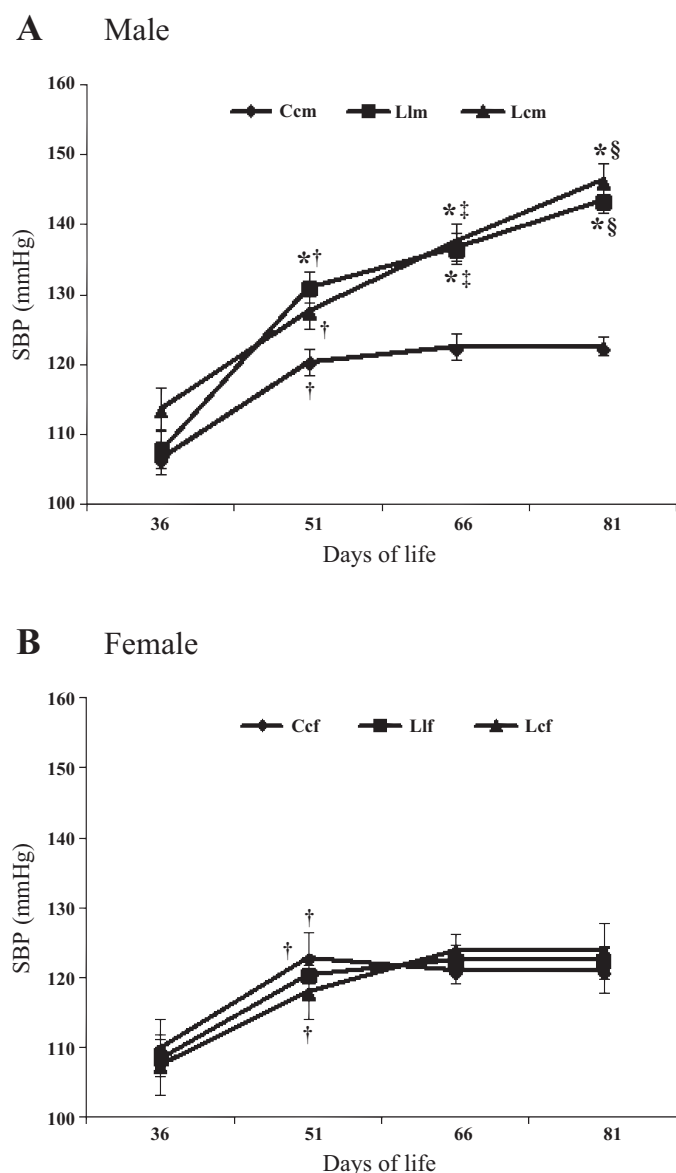


Fig. 1. A: systolic blood pressure (SBP) measured in control diet-fed male offspring of control diet-fed mothers (Ccm group), low-zinc diet-fed male offspring of low-zinc diet-fed mothers (Llm group), and control diet-fed male offspring of low-zinc diet-fed mothers (Lcm group) at 36, 51, 66, and 81 days of life. B: SBP of control diet-fed female offspring of control diet-fed mothers (Ccf group), low zinc-diet-fed female offspring of low-zinc diet-fed mothers (Llf group), and control diet-fed female offspring of low-zinc diet-fed mothers (Lcf group) at 36, 51, 66, and 81 days of life. Values are means  $\pm$  SE;  $n = 10$  animals/group. Data were analyzed using two-way ANOVA followed by a Bonferroni post hoc test. Factors of diet and time were considered significant at  $*P < 0.01$  vs. the Ccm group,  $\dagger P < 0.001$  vs. 36 days of life,  $\ddagger P < 0.05$  vs. 51 days of life,  $\S P < 0.05$  vs. 66 days of life; interaction time  $\times$  diet: not significant and interaction sex  $\times$  diet: not significant.

ters among Llf, Lcf and Ccf rats. Heart rate was similar in all male and female groups studied.

**Histological evaluation of LV tissue.** Figure 2, A and B, shows MCD measured in the LV at 6, 21, and 81 days. All dietary groups showed a lifetime increase in MCD. In early postnatal life (6 days), only the Lf group exhibited larger MCD than the Cf group. At weaning (21 days), MCD was larger in Lm rats than in Cm rats. However, in adult life (81 days), MCD

was smaller in the Llm and Llf groups compared with the Ccm and Ccf groups, respectively. An adequate diet after weaning prevented this alteration at 81 days of life. Picrosirius red staining showed an increase in ICF between 6 and 21 days and a decrease of ICF between 21 and 81 days of life in all studied groups. However, there were no signs of early fibrosis in the LV of male and female offspring exposed to zinc deficiency during fetal life, lactation, and/or postweaning growth at any aged studied (Fig. 3).

Examination of TUNEL-stained LV sections revealed a large increase in the number of apoptotic cells in Lm and Lf offspring at 6 days of life compared with Cm and Cf offspring, respectively. However, the number of apoptotic cells was similar in all groups at 21 days of life (Fig. 4).

There were no differences in MCD, collagen-specific picrosirius red-stained areas, and number of apoptotic cells in the LV between male and female control rats.

**Coronary artery morphology.** PVC/LA, CWA/LA, and LA/TVA were similar in Ccm and Llm rats at 6 and 21 days of life, whereas differences in these parameters were found in the male dietary groups at 81 days of life. LA/TVA was diminished in coronary arteries of Llm and Lcm offspring compared with Ccm offspring. However, CWA/LA was greater in Llm and Lcm groups compared with the Ccm group, and PVC/LA was greater in the Llm group than in Ccm and Lcm groups (Figs. 5, A, C, and E, and 6). However, there were no differences in these morphology parameters between the Ccf and Llf groups at 6 and 21 days or among Ccf, Llf, and Lcf groups at 81 days (Figs. 5, B, D, and F, and 6).

## DISCUSSION

The results of the present study demonstrate that moderate zinc deficiency during critical periods of development leads to permanent and long-term changes in heart structure, which result in the programming of a reduced functional capacity of this organ for life and predispose to CVD in adult life.

Male and female offspring exposed to moderate zinc deficiency showed a growth delay. However, males would be most affected by this deficiency as they exhibited lower heart weights than the control groups, whereas this was compensated in females in adult life. Moreover, the fact that an adequate zinc diet during postweaning life could not normalize these growth markers highlights the importance of this micronutrient during fetal life and lactation in the development of organs and systems. In this regard, several studies have reported that zinc stimulates cell proliferation through the functional or synthesis dependency on zinc of various cell division- and proliferation-related enzymes and hormones. For example, zinc upregulates gene expression of the DNA-synthesizing enzyme deoxythymidine kinase (21) and stimulates the synthesis of the growth hormone and insulin-like growth factors (9).

As in our previous studies, we observed that zinc-deficient male offspring exhibited an increase in SBP from 51 days of life, exhibiting values higher than 140 mmHg on day 81. However, this alteration could not be prevented by an adequate zinc diet after weaning. This degree of blood pressure elevation dramatically increases the risk of cardiovascular events (40, 41, 42, 44, 48). Moreover, in the present study, we demonstrated, for the first time, that fetal and postnatal zinc restriction does not modify blood pressure levels in females. These results are

Table 3. Echocardiographic analysis of the heart at 81 days of life

	Ccm Group	Llm Group	Lcm Group	Ccf Group	Llf Group	Lcf Group
LVIDs, mm	2.8 ± 0.1	4.3 ± 0.2*	3.4 ± 0.1*†	3.3 ± 0.2	3.0 ± 0.2	3.3 ± 0.2
PWTs, mm	3.5 ± 0.2	2.6 ± 0.1*	3.0 ± 0.1*	3.2 ± 0.3	2.5 ± 0.3	2.7 ± 0.3
AWTs, mm	3.3 ± 0.1	2.4 ± 0.1*	2.7 ± 0.1*	2.8 ± 0.1	2.6 ± 0.1	2.5 ± 0.1
LVIDd, mm	5.4 ± 0.1	6.3 ± 0.1*	5.8 ± 0.2	5.5 ± 0.2	4.9 ± 0.2	4.8 ± 0.3
PWTd, mm	2.7 ± 0.1	2.1 ± 0.1*	2.3 ± 0.1	2.4 ± 0.1	2.1 ± 0.1	2.3 ± 0.2
AWTd, mm	2.3 ± 0.1	1.8 ± 0.1*	1.7 ± 0.1*	1.9 ± 0.1	1.8 ± 0.1	1.9 ± 0.1
RWT	0.93 ± 0.05	0.61 ± 0.04*	0.69 ± 0.06*	0.80 ± 0.04	0.80 ± 0.03	0.88 ± 0.08
EF, %	83 ± 3	65 ± 4*	78 ± 3†	78 ± 1	76 ± 3	72 ± 3
FS, %	48 ± 3	32 ± 2*	42 ± 3†	39 ± 1	39 ± 2	35 ± 2
HR, beats/min	490 ± 6	463 ± 20	432 ± 12	464 ± 26	447 ± 26	416 ± 19

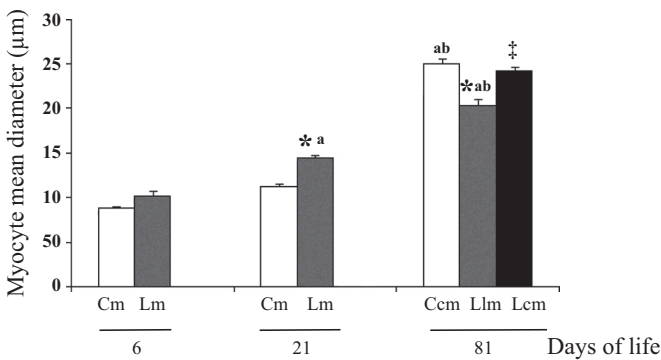
Values are means ± SE; n = 10 animals/group. The following groups are shown: Ccm, Llm, Lcm, Ccf, Llf, and Lcf. Left ventricular internal diameter (LVID), left ventricular posterior wall thickness (PWT), and anterior wall thickness (AWT) were measured at both systole (s) and diastole (d). Relative left ventricular wall thickness (RWT), ejection fraction (EF), and fractional shortening (FS) were measured from ventricular internal diameters and wall thickness. Heart rate (HR) was monitored by ECGs. The factor of diet was considered significant: \*P < 0.01 vs. the Ccm group and †P < 0.01 vs. the Llm group.

consistent with findings across diverse animal models examining the effects of altered in utero environments. In rats, hypertension programmed in response to placental insufficiency results in hypertension in growth-restricted male, but not female, offspring (26, 27). In sheep, blood pressure is

higher in male offspring after fetal exposure to glucocorticoids (10). Therefore, sex differences in developmental programming are observed in female offspring, which exhibit a protected status regardless of the species or specific fetal insult.

On the other hand, the impact of this nutritional injury on heart morphology changed over life and was more marked in males than in females. In early postnatal life, Lf and Lm groups

**A Males**



**B Females**

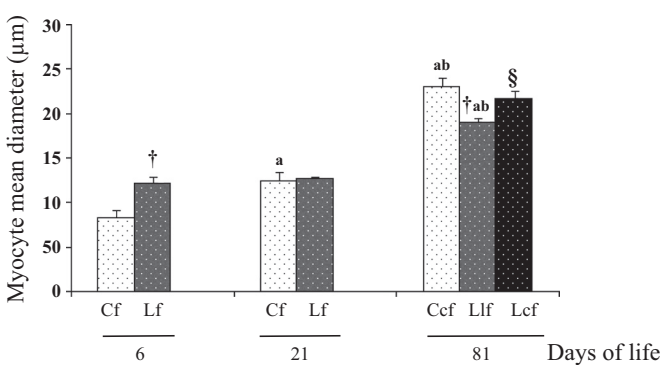
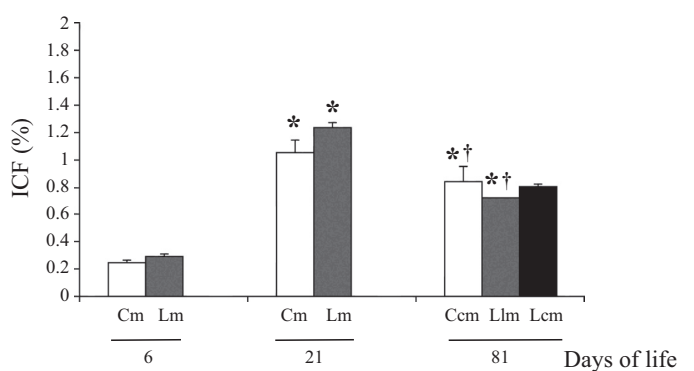


Fig. 2. Mean cardiomyocyte diameter measured in left ventricular (LV) cross-sections at 6, 21, and 81 days in male (A) and female (B) offspring. The following zinc diet groups are shown: Cm, Lm, Cf, Lf, Ccm, Llm, Lcm, Ccf, Llf, and Lcf. Values are means ± SE; n = 10 animals/group. Data were analyzed using two-way ANOVA followed by a Bonferroni post hoc test. Factors of diet and time were considered significant at \*P < 0.001 vs. the Cm/Ccm group, †P < 0.001 vs. the Cf/Ccf group, ‡P < 0.001 vs. the Llm group, §P < 0.001 vs. the Llf group, <sup>a</sup>P < 0.001 vs. 6 days, and <sup>b</sup>P < 0.01 vs. 21 days; interaction time × diet: not significant and interaction sex × diet: not significant.

**A Males**



**B Females**

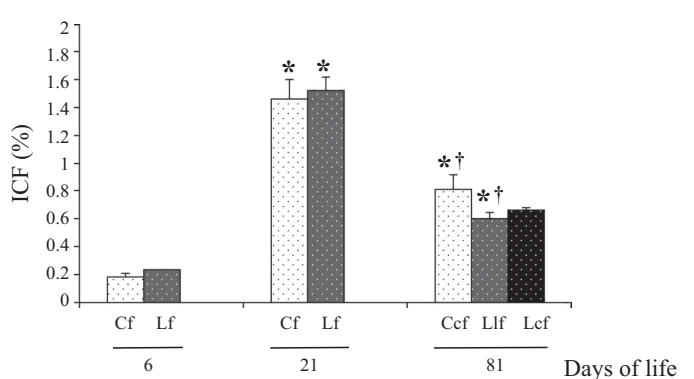


Fig. 3. Interstitial collagen fraction (ICF) expressed as a percentage of the total LV area at 6, 21 and 81 days in male (A) and female (B) offspring. The following zinc diet groups are shown: Cm, Lm, Cf, Lf, Ccm, Llm, Lcm, Ccf, Llf, and Lcf. Values are means ± SE; n = 10 animals/group. Data were analyzed using two-way ANOVA followed by a Bonferroni post hoc test. Factors of diet and time were considered significant at \*P < 0.001 vs. 6 days and †P < 0.01 vs. 21 days; interaction time × diet: not significant.

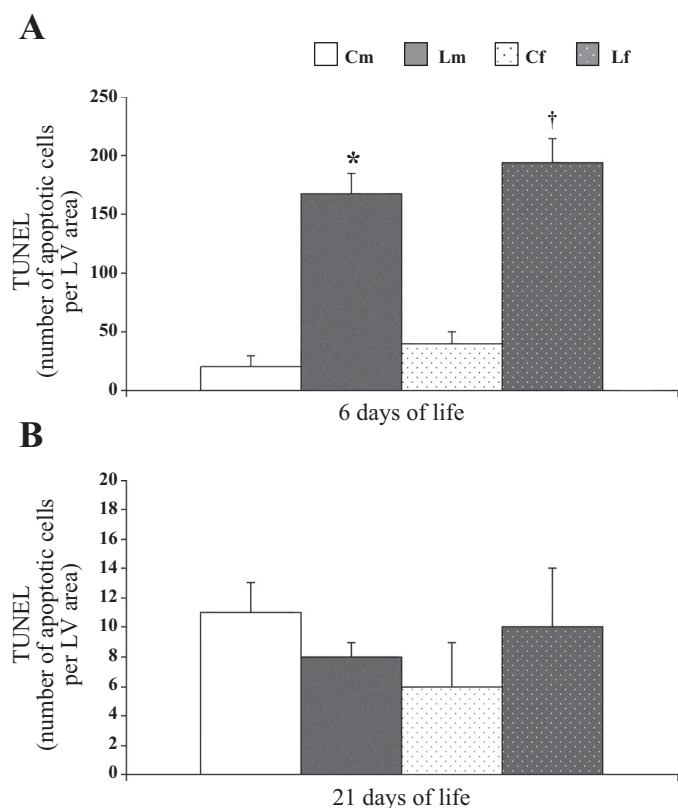


Fig. 4. Numbers of TUNEL-positive cells per LV area at 6 days (A) and 21 days (B) in male and female offspring. The following zinc diet groups are shown: Cm, Lm, Cf, and Lf. Values are means  $\pm$  SE;  $n = 6$  animals/group. Data were analyzed using two-way ANOVA followed by a Bonferroni post hoc test. The factor of diet was considered significant at 6 days of life: \* $P < 0.001$  vs. the Cm group and † $P < 0.001$  vs. the Cf group; interaction diet  $\times$  sex: not significant.

showed an increase in the mean diameter of myocytes, which became evident in female rats first. This remodeling of myocytes could be an adaptive response to inadequate cardiac development induced by zinc restriction. In rats, there is a transition period during the first week of life when the increase in myocardial mass induced by hyperplasia of cardiomyocytes is replaced by hypertrophy (18). Previous studies have reported that this transition is regulated by controlled proliferation and apoptosis of cardiomyocytes (33) and may further be influenced by nutritional, hemodynamic, and humoral factors (7, 24, 47). At 21 days, cardiac growth is mainly due to hypertrophy of cardiomyocytes and extracellular matrix deposition rather than apoptotic mechanisms (33). These findings support our results showing differences in cardiac apoptosis between days 6 and 21. Therefore, we suggest that prenatal and early postnatal zinc deficiency could affect myocardial development in the first week of life since zinc-deficient offspring exhibited an abrupt increase in apoptotic cardiac cells at 6 days of life. However, a sex difference in the impact of this nutritional injury on other mechanisms, such as cell division and proliferation, could explain, in part, the subsequent adaptive increase in myocyte diameter observed in male hearts. Therefore, further studies are needed to clarify this query.

On the other hand, this adaptive response would not remain through adulthood since male and female rats exposed to zinc

deficiency throughout their life had smaller myocyte diameter in adult life. Moreover, no signs of fibrosis were observed in the LV of male and female offspring exposed to zinc deficiency at any of the ages studied. Therefore, morphological alterations of the functional units of the heart observed in adult life were already present in early stages of life in both male and female rats. Similar effects of zinc deficiency during fetal life, lactation, and postweaning growth have been previously observed in renal glomeruli (42, 43).

The pattern of cardiac myocyte remodeling and the lower heart weight observed in zinc-deficient male offspring during adult life correlates well with the changes in LV chamber anatomy and function determined by echocardiography. The smaller RWT and LV wall thicknesses as well as the larger LV diameter observed in the Llm group revealed a decreased contractility in systole that could lead to a dilatation of this chamber during diastole. Moreover, the fact that EF and FS were reduced supports the fact that this group of animals exhibited impaired LV contractile function. These results demonstrate that dietary zinc restriction in male rats throughout life induces changes in ventricular function and structure, which does not allow the heart to compensate the increase in pressure overload induced by higher levels of blood pressure. On the other hand, female cardiac tissue would be less affected by this nutritional insult during development. Echocardiographic studies have shown that zinc-deficient females exhibit no significant structural heart alterations.

Furthermore, the reinstatement of adequate zinc content during postweaning life in male and female offspring could not normalize the decrease in body weight induced by zinc deficiency during fetal life and lactation. However, this could overcome heart weight and myocyte diameter and partially prevented the alterations in LV wall thicknesses and diameter during diastole and systole described in Llm rats. Moreover, these changes were sufficient to ensure adequate EF and FS. Therefore, Lcm animals would be better able to compensate for the increase in blood pressure.

In zinc-deficient adult male rats, the structural and functional alterations of the LV were accompanied by hypertrophic remodeling of coronary artery architecture. Arterial remodeling is thought to reflect adaptation of the vessels to mechanical and hemodynamic stimuli. Therefore, we propose that the chronic elevation in blood pressure in Llm and Lcm animals could cause wall tightening and, consequently, a reduction of the inner lumen and an increase in the peripheral resistance of these vessels. Moreover, increased perivascular collagen deposition in Llm adult offspring might alter the pressure-diameter relation of arteries at higher pressures. On the other hand, normotensive zinc-deficient female offspring showed no signs of coronary artery remodeling. Furthermore, the increase in lumen coronary area accompanying growth in Ccm and Ccf offspring would allow adequate coronary blood flow supply for heart function in adult life.

The results of the present study demonstrate that the heart would not be the main responsible for the increase in blood pressure observed in Llm and Lcm offspring. Therefore, we postulate that the impact of zinc deficiency during fetal life, lactation, and/or postweaning growth on other organs, such as the kidneys and blood vessels, could contribute to the increased arterial blood pressure in males. In previous studies, we demonstrated that dietary zinc restriction during these periods of

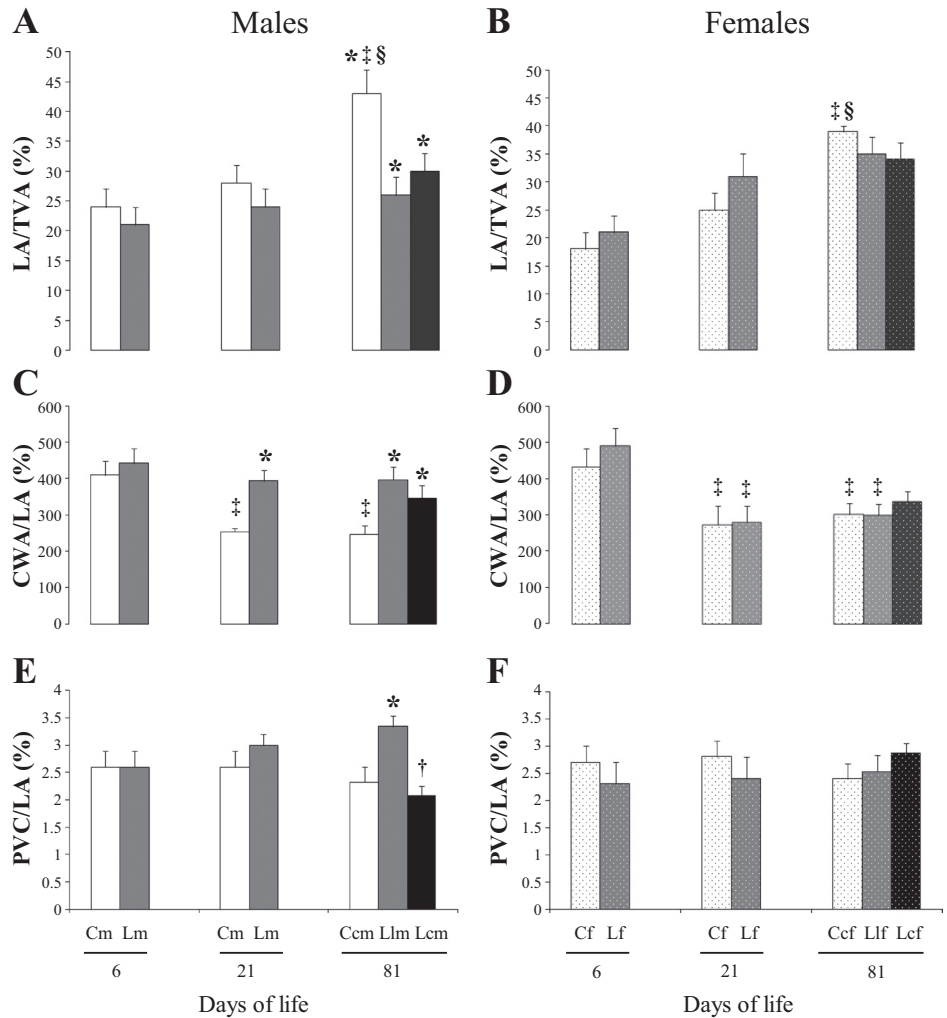


Fig. 5. Coronary artery morphology evaluated in LV cross-sections at 6, 21, and 81 days in male (A, C, and E) and female (B, D, and F) offspring. A and B: lumen area (LA)/total vascular area (TVA). C and D: coronary artery wall area (CWA)/LA. E and F: perivascular collagen (PVC)/LA. The following zinc diet groups are shown: Cm, Lm, Cf, Lf, Ccm, Llm, Lcm, Ccf, Llf and Lcf. Values are means ± SE; n = 10 animals/group. Data were analyzed using two-way ANOVA followed by a Bonferroni post hoc test. Factors of diet and time were considered significant at \*P < 0.01 vs. the Cm/Ccm group, †P < 0.01 vs. the Llm group, ‡P < 0.001 vs. 6 days, and §P < 0.01 vs. 21 days; interaction time × diet: not significant and interaction sex × diet: not significant.

life impairs renal function in adult life. Among renal and vascular alterations, zinc-deficient rats showed a decrease in the glomerular filtration rate associated with a reduction in nephron number and glomerular filtration surface areas, nitric oxide system impairment, and an increase in renal oxidative stress, apoptosis, fibrosis, and proteinuria (40, 42).

Moreover, we posit that the lower LV structural and functional capacity would be mainly a consequence of inadequate development of the cardiovascular system induced by moderate zinc restriction during prenatal and postnatal life. In this regard, we would expect chronic increment in SBP to lead to LV hypertrophy and an increase in cardiac contractility to better for compensate pressure overload in adult life. Structural and functional alterations in cardiac tissue have also been reported in other animal models of fetal programmed hypertension. A previous study (3) has demonstrated that rats exposed to glucocorticoid show permanent histopathological changes during growth and development of the heart, and they exhibit smaller hearts with larger cardiomyocytes and additional cardiomyocyte loss during adulthood. Vitamin D deficiency in early life increases arterial blood pressure and induces changes in several genes involved in the regulation of oxidative stress and myocardial hypertrophy (2). Further, another study (6) has shown that male rats exposed to fetal and

postnatal growth restriction had a lower cardiomyocyte numbers in early postnatal life and elevated blood pressure not accompanied by cardiac hypertrophy at 6 mo of age. These conflicting results indicate that the programming of blood pressure and cardiac function alterations may be related to the type, duration, and degree of injury as well as the period of life involved (pregnancy, fetal life, weaning, childhood, and adulthood).

The present study also shows that male and female offspring adapt differently to developmental stressors, with female offspring exhibiting a protected cardiovascular status. This sex-specific effect is consistent with previous findings in rats in which intrauterine growth restriction, as a result of either hypoxia or a low-protein diet, caused cardiac remodeling and impaired recovery to ischemia-reperfusion in adult male offspring but had no effect on the female heart (15). Moreover, it has been shown that chronic hypoxia in early intrauterine development induced increased end-diastolic volume in both male and female adult rats, but female rats exhibited improved contractility and increased coronary flow, whereas male rats showed little compensation (34, 50). Considering that the main sex differences in blood pressure and cardiac structure were found after weaning and in adult life, respectively, we postulate that sex hormones could influence the development of the

cardiovascular alterations induced by this nutritional injury. It is known that male and female sex steroids have a profound influence on the development and progression of programmed disease states. Testosterone has been demonstrated to be a key factor in hypertension programming in growth-restricted male offspring, whereas estradiol has been implicated to play a protective role against hypertension in growth-restricted female offspring. Moreover, innate sex differences and epigenetic changes, as well as changes in both gene expression associated with sex chromosomes and the activity of systems modulated by sex hormones, could explain the differences found in our experimental model (25–27). In these regard, estrogen has been demonstrated to modulate the renin-angiotensin-aldosterone system and to have beneficial effects on cardiovascular function through actions in the kidney, heart, vasculature, and central nervous system (30, 36). Moreover, cardiac sex differences induced by zinc deficiency could also be mediated by changes in oxidative stress and in the nitric oxide system. Therefore, further research needs to be conducted to elucidate the mechanisms involved in sex-related differences in response to zinc deficiency.

In conclusion, the results of the present study provide strong evidence showing that zinc deficiency during prenatal and postnatal life could alter the normal trajectory of cardiac development. The consequences of this micronutrient imbalance on the developing fetus would induce adaptive responses of cardiac myocytes in early postnatal life that would become manifest later in adulthood. In males, this nutritional injury induced decreased contractility and dilatation of the LV, which would not allow the heart to compensate the higher levels of blood pressure that these animals showed in adult life. Moreover, zinc-deficient adult males showed hypertrophic remodeling of the coronary artery architecture associated with this chronic increase in arterial blood pressure. On the other hand, females would be less sensitive to this micronutrient deficiency since they exhibited no significant structural or functional heart alterations. Moreover, adequate zinc content in the diet during postnatal life could reverse some of the detrimental effects of this earlier micronutrient deficiency on cardiac tissue.

The data strengthen the importance of prenatal and postnatal care optimization for better management and prevention of CVD. Future investigations will be performed to evaluate the

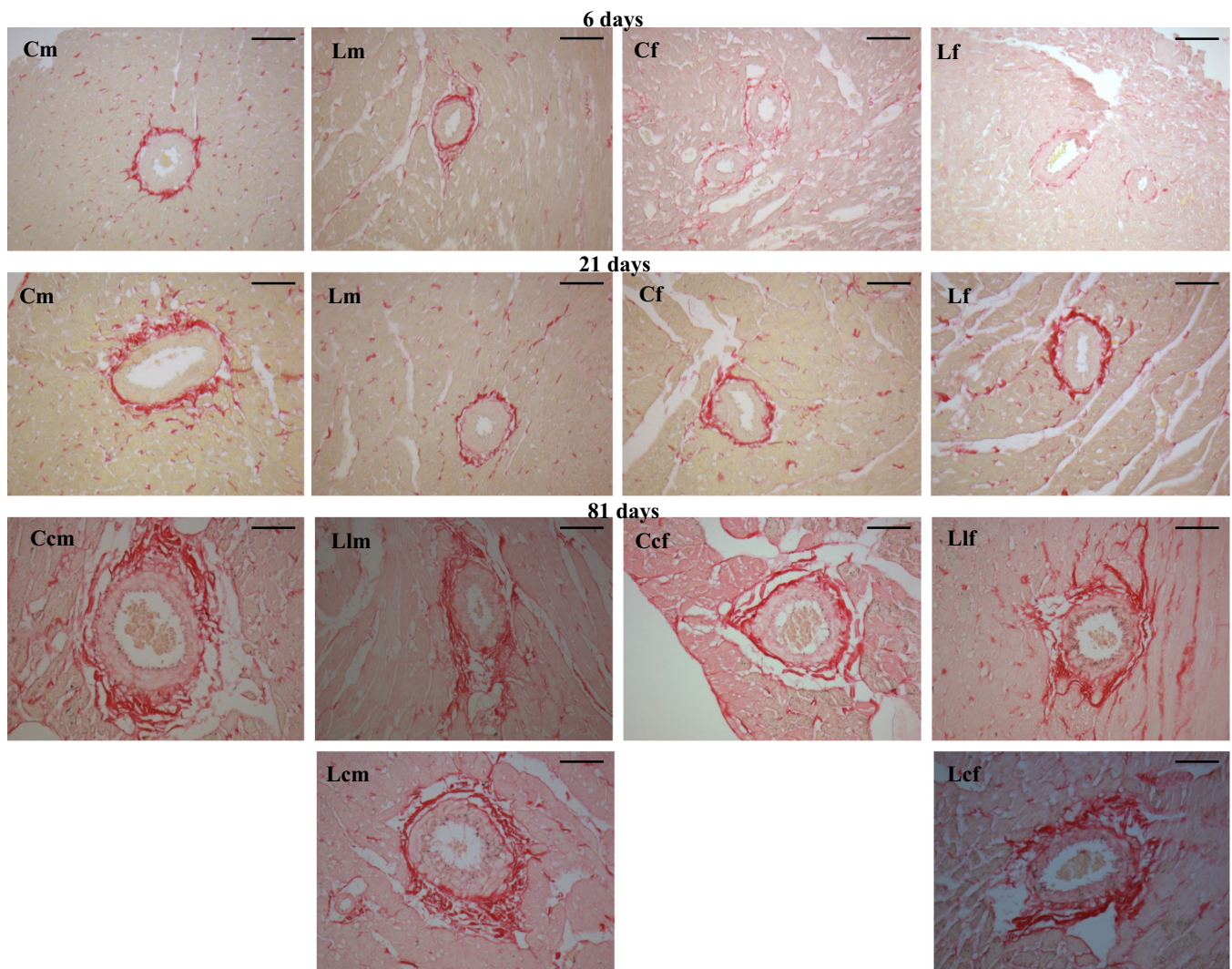


Fig. 6. Sirius red staining in LV coronary arteries of Cm, Lm, Cf, Lf, Ccm, Lcm, Lcm, Ccf, Llf, and Lcf animals at 6, 21, and 81 days. All images are at the same magnification ( $\times 400$ ). Scale bar = 30  $\mu\text{m}$ .



possible mechanisms responsible for the alterations in cardiac function and morphology observed in fetal programming induced by zinc deficiency.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### AUTHOR CONTRIBUTIONS

Author contributions: A.L.T., M.A.C., and C.A. conception and design of research; A.L.T., L.V.J., L.C.V., F.M.G.A., J.Z., H.F., R.E., M.A.C., and C.A. analyzed data; A.L.T., L.V.J., J.Z., M.A.C., and C.A. interpreted results of experiments; A.L.T. and R.E. prepared figures; A.L.T., L.V.J., M.A.C., and C.A. drafted manuscript; A.L.T., M.A.C., and C.A. edited and revised manuscript; A.L.T., L.V.J., M.N.G., L.C.V., F.M.G.A., J.Z., H.F., R.E., M.A.C., and C.A. approved final version of manuscript; L.V.J., M.N.G., L.C.V., F.M.G.A., J.Z., H.F., and R.E. performed experiments.

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