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Original Paper

Zinc Supplementation Attenuates Cardiac Remodeling After Experimental Myocardial Infarction

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Key Words
Coronary occlusion • Function • Hypertrophy • Chronic remodeling • Zinc • Treg cells

Abstract
Background/Aims: the objective of our study was to evaluate the effects of zinc supplementation on cardiac remodeling following acute myocardial infarction in rats. ***Methods:*** Animals were subdivided into 4 groups and observed for 3 months: 1) Sham Control; 2) Sham Zinc: Sham animals receiving zinc supplementation; 3) Infarction Control; 4) Infarction Zinc. After the followup period, we studied hypertrophy and ventricular geometry, functional alterations *in vivo* and *in vitro*, changes related to collagen, oxidative stress, and inflammation, assessed by echocardiogram, isolated heart study, western blot, flow cytometer, morphometry, and spectrophotometry. ***Results:*** Infarction induced a significant worsening of the functional variables. On the other hand, zinc attenuated both systolic and diastolic cardiac dysfunction induced by infarction. Considering the infarct size, there was no difference between the groups. Catalase and superoxide dismutase decreased in infarcted animals, and zinc increased its activity. We found higher expression of collagens I and III in infarcted animals, but there was no effect of zinc supplementation. Likewise, infarcted animals had higher levels of IL-10, but without zinc interference. Nrf-2 values were not different among the groups. Infarction increased the amount of Treg cells in the spleen as well as the amount of total lymphocytes. Zinc increased the amount of CD4+ in infarcted animals, but we did not observe effects in

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relation to Treg cells. ***Conclusion:*** zinc attenuates cardiac remodeling after infarction in rats; this effect is associated with modulation of antioxidant enzymes, but without the involvement of collagens I and III, Nrf-2, IL-10, and Treg cells.

Introduction

The process of cardiac remodeling after myocardial infarction is associated with a poor prognosis. In the acute phase, remodeling favors the formation of the aneurysm and predisposes the infarcted heart to ventricular rupture. Chronically, it is associated with a higher prevalence of malignant arrhythmias and ventricular dysfunction, as the noninfarcted region undergoes genetic, structural, and biochemical changes that will result in deterioration of the heart's functional capacity and death [1-5]. Therefore, strategies that attenuate remodeling result in improved postinfarction prognosis.

Recently, the role of zinc as a modulator of the cardiac remodeling process has been the object of interest of several studies [6-16]. Among the mechanisms suggested for the influence of zinc on cardiac remodeling, matrix metalloproteinases activity, increased oxidative stress, and inflammatory process seem to play a critical role.

An important issue is that the role of zinc supplementation on cardiac remodeling after myocardial infarction remains unknown. Therefore, the objective of our study was to evaluate the effects of zinc supplementation following acute myocardial infarction in rats by studying some of the main mechanisms involved in the cardiac remodeling process: hypertrophy and ventricular geometry, functional alterations *in vivo* and *in vitro*, changes related to collagen, oxidative stress, and inflammation.

Materials and Methods

This research protocol was approved by the Animal Ethics Committee of Botucatu Medical School, and it was performed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Animals and Groups

Male Wistar rats, weighing 200–250 g, were subjected to experimental myocardial infarction, according to the method described previously [17, 18] or a simulated surgery (without coronary artery occlusion).

The animals were housed in cages with 2 or 3 animals, with free access to water and feed. The temperature was maintained at 22 ± 2°C, and the humidity was controlled. Light cycles (light/dark) were 12 hours. The animals were weighed once a week throughout the experimental period.

We selected only animals with an infarct size greater than 35% as assessed by histologic analysis, because we considered that animals with small infarct size do not undergo cardiac remodeling [19].

After 3 to 5 days of performing the surgical procedure of infarction induction, an initial echocardiographic study was performed to evaluate systolic and diastolic areas and fraction of area variation in all animals, in addition to the measurement of infarct size in animals induced by occlusion of the coronary artery, to guarantee homogeneity between the groups (data not shown).

After the initial echocardiographic study, the animals were again subdividedinto 4 experimental groups and observed for 3 months: 1) Sham Control (SC): Sham animals that received intraperitoneal saline solution on alternate days; 2) Sham Zinc (SZ): Sham animals receiving zinc supplementation (zinc sulfate, 5 mg/kg) intraperitoneally on alternate days; 3) Infarction Control (IC): Infarcted animals receiving intraperitoneal saline solution on alternate days; 4) Infarction Zinc (IZ): Infarcted animals receiving zinc supplementation (zinc sulfate, 5 mg/kg) intraperitoneally on alternate days. The zinc dose used in our study was based on a previous study, which showed beneficial effects of this dose for preventing diabetic heart disease [20].

Echocardiographic analysis

After 3 months, all the rats were weighed and evaluated by a transthoracic echocardiographic examination (General Electric Medical Systems, Vivid S6, Tirat Carmel, Israel). The following structural variables were measured: left atrium diameter, left ventricle (LV) diameter, diastolic and systolic dimensions, and LV diastolic posterior wall thickness. Systolic function was assessed based on the fractional area change (FAC), Tei index, and posterior wall shortening velocity (PWSV). The E/E' ratio and LA diameter were used as a variable of LV diastolic function [21].

Isolated heart study: Langendorff preparation

After 3 months of exposure, six animals from each group were anesthetized with thiopental (80 mg/kg, IP) and received unfractionated heparin (2, 000 IU, IP). After sternotomy, the rats were artificially ventilated, and the ascending aorta was dissected and cannulated. Retrograde perfusion was initiated with a modified Krebs-Henseleit solution, constantly gassed with a mixture of 95% O_2 and 5% CO_2, and a perfusion pressure of 75 mmHg [22]. The heart was removed and transferred to an isolated heart perfusion apparatus (size 3, type 830, Hugo Sachs Elektronik - March-Hugstetten, Germany). A balloon was inserted in the LV cavity, and the volume inside the balloon was modified to obtain a diastolic pressure of zero at 25 mmHg. We registered the diastolic and systolic pressures, the maximum LV pressure decrease rate (-dP/dt) and the maximum LV pressure development rate (+dP/dt). Systolic function was evaluated by systolic pressure and +dP/dt, and diastolic function by -dP/dt. The hearts that were subjected to the isolated heart study were not utilized for any other analysis because retrograde perfusion can interfere with subsequent biochemical analysis.

Morphometric analysis

On completion of the echocardiographic study, the right and left ventricles (including the interventricular septum) were dissected, separated, and weighed. The lengths of the infarcted and the viable muscle for both the endocardial and epicardial circumferences were determined by planimetry. Infarct size was calculated by dividing the endocardial and epicardial circumferences of the infarcted area by the total epicardial and endocardial ventricular circumferences. The measurements were performed on ventricular sections (5-6 mm from the apex) under the assumption that the left mid-ventricular slice showed a close linear relationship with the sum of the area measurements from all the heart sections [23, 24].

Antioxidant enzyme analysis

LV samples (100 mg) were homogenized in 5 ml of 0.1 M cold sodium phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid. Tissue homogenates were prepared, and total protein concentration, glutathione peroxidase, superoxide dismutase (SOD), and catalase were assessed as previously specified [25]. All reagents were from Sigma (St. Louis, MO, USA).

Western blot analysis for Nrf-2, IL-10, and type I and III collagen

Samples of the LV were homogenized in RIPA buffer and diluted in Laemmli buffer to detect type I collagen (rabbit polyclonal IgG, sc8784R; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and type III collagen (mouse monoclonal IgG1, ab6310; Abcam, Inc., Cambridge, UK). Nuclear protein extraction from the LV was performed with the NE-PER Nuclear Extraction Reagents kit (Pierce Biotechnology, Rockford, IL, USA). Nuclear extracts were used to detect Nrf-2 (C-20, rabbit polyclonal IgG, sc722; Santa Cruz Biotechnology, Inc.). Secondary antibodies were used according to the manufacturer's recommendations, and GAPDH (GAPDH [6C5], mouse monoclonal IgG1, sc32233, Santa Cruz Biotechnology, Inc.) was used for normalization [26].

Determination of regulatory T cells (Treg) in the spleen

The spleen was macerated with 1 ml of incomplete RPMI medium, and this cell suspension was placed in a 15 ml Falcon tube and the volume filled with incomplete RPMI medium (with gentamicin) to 7 ml and then centrifuged at 300g, for 10 minutes, at 4°C. The supernatant was discarded, and the red cells were lysed by resuspending the spleen pellet in 5 ml of lysis buffer and incubated for 5 minutes at room temperature. Volume was completed with incomplete RPMI medium up to 15 ml and centrifuged again at

300 g, for 10 minutes, at 4°C. The supernatant was discarded, and the cells resuspended in 1 ml of complete RPMI medium (10% FBS and 1% L-glutamine). The number of cells was counted, with Turk dye.

CD4 and CD25 were labeled in a tube for light-protected cytometry (laminated paper). A total of 2.5 x 10^6 cells were used in a total volume of 100 µl + 0.5 µg (1 µl) CD4-FITC antibody + 0.25 µg (1.3 µl) CD25-APC antibody, incubated for 20 minutes at room temperature. For the isotype control, 75 µl of cells (2.5 × 10^6 cells) + 20 µl (1 µg) anti-FITC + 5 µl (0.5 µg) anti-APC were used. After labeling, 1 ml of AutoMacs Running Buffer - Miltenyi Biotec buffer was centrifuged at 450 *g*, for 10 minutes, at 4°C. The supernatant was discarded and then labeled with Foxp3.

For labeling Foxp3, the pellet was resuspended, and 1 ml of permeabilization/fixation buffer was added and then incubated at 4°C in the dark. After washing and centrifugation steps, 1 µl (0.2 µg) of anti-Foxp3-PE antibody was added. For the isotype control, 95 µl permeabilization buffer + 5 µl anti-PE was used. Incubation was performed in the dark. After washing and further centrifugation, the samples were read on a flow cytometer.

Zinc dosage

The blood of animals was collected and centrifuged at 3, 000 rpm for 20 minutes at 4°C, and the serum stored at -80°C. Subsequently, in a small fraction of the serum, the zinc dosage was made by atomic absorption spectrophotometry.

Statistical analysis

The data are represented as mean and standard deviation. Variables with non-normal distributions were normalized before comparisons. A two-way ANOVA test complemented by the Holm-Sidak test was used to compare the groups. For mortality comparison, we used the chi-square test. Data analysis was performed with SigmaStat for Windows v2.03 (SPSS, Inc., Chicago, IL, USA). The significance level was 5%.

Table 1. Echocardiographic Data. SC=sham animals that received standard diet; SZ=sham animals that received diet with zinc supplementation; IC= infarcted animals that received standard diet; IZ= infarcted animals that received diet with zinc supplementation. LVDD: left ventricular diastolic diameter; LVSD: left ventricular systolic diameter; LVWT: posterior wall shortening velocity; FAC: fractional area change; SA: systolic area; DA: diastolic area. Data are expressed as the mean ± standard error of the mean. n=number of rats. p(I)=p value of the I effect; p(VZ)=p value of the zinc effect; p(IxZ)=p value of their interaction

Variables	SC (n=15)	SZ (n=15)	IC (n=23)	IZ (n=24)	p(I)	p(Z)	p(IxZ)
LVDD (mm)	7.5±0.2	7.0±0.3	9.4±0.2	9.0±0.2	<0.001	0.079	0.874
LVSD (mm)	3.7±0.4	3.1±0.4	6.7±0.2	6.2±0.2	<0.001	0.159	0.925
LA (mm)	5.5±0.1	4.9±0.1	6.2±0.1	5.7±0.1	<0.001	0.028	0.524
FAC (%)	57.8±12.9	73.8±12.8	24.2±8.9	43.9±8.8	0.003	0.091	0.899
Tei index	0.63±0.06	0.45±0.07	0.94±0.04	0.82±0.04	<0.001	0.011	0.599
LVWT (mm/s)	55.6±2.5	64.0±2.4	36.1±1.5	39.2±1.6	<0.001	0.010	0.245
E/E'	13.2±1.0	11.9±1.1	17.6±0.7	15.5±0.7	<0.001	0.050	0.598
SA (mm^2)	22.6±3.7	11.2±3.6	50.1±2.6	44.0±2.6	<0.001	0.007	0.401
DA (mm^2)	50.8±4.7	40.1±4.6	79.4±3.2	73.9±3.2	<0.001	0.051	0.430

Results

After 48 hours of surgery, the mortality of infarcted animals was 27.7%, with 125 surviving animals. In the noninfarcted group, all animals survived. During the follow-up period, 7 animals from the IC group and 6 animals from the IZ group died (p>0.05). As previously mentioned, we used only animals with infarctions greater than 35%, thus totaling the number of animals indicated in the tables.

Echocardiogram results are shown in Table 1. As expected, infarction induced a significant worsening of the functional variables. On the other hand, zinc attenuated both systolic and diastolic cardiac dysfunction induced by infarction, assessed by Tei index, LVWT, LA diameter, and E/E' ratio.

In the study of the isolated heart, we found alterations in infarcted animals, with a decrease in the positive (SC (n=8)=2725±222 mmHg/s, SZ (n=8)=2850±232 mmHg/s, IC (n=7)=1350±253 mmHg/s, IZ (n=7)=1450±222 mmHg; p(i)<0.001, p(z)=0.541, p(iz)=0.542) and negative (SC (n=8)=1675±131 mmHg/s, SZ (n=8)=1875±141 mmHg/s, IC (n=7)=875±155 mmHg/s, IZ (n=7)=975±131 mmHg; p(i)<0.001, p(z)=0.555, p(iz)=0.880) derivatives as well as in the systolic pressure (SC (n=8)=163±7.4 mmHg, SZ n=8)=153±7.2 mmHg, IC (n=7)=91±8.1 mmHg, IZ (n=7)=93±8.9 mmHg; p(i)<0.001, p(z)=0.660, p(iz)=0.705), but without the influence of zinc .

Considering the infarct size, there was no difference between the groups (IC = 42.6% ± 6.5, IZ = 40.6% ± 5.3; p = 0.691).

The activity of enzymes related to oxidative stress are presented in Table 2. Catalase decreased in infarcted animals, and zinc increased its activity. Considering the enzyme SOD, the infarcted animals that received zinc supplementation had lower SOD activity than the infarcted control. Glutathione peroxidase decreased in infarcted animals, and zinc increased its activity.

Table 3 shows the protein expression levels. We found higher expression of collagens I and III in infarcted animals, but there was no effect of zinc supplementation. Likewise, infarcted animals had higher levels of IL-10, but without zinc interference. Nrf-2 values were not different among the groups.

The leucocyte results are shown in Table 4. Infarction increased the amount of Treg cells

Table 2. Cardiac Oxidative Stress Enzyme Activity. SC=sham animals that received standard diet; SZ=sham animals that received diet with zinc supplementation; IC= infarcted animals that received standard diet; IZ= infarcted animals that received diet with zinc supplementation. CAT: catalase; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase

Variables	SC (n=10)	SZ (n=10)	IC (n=10)	IZ (n=10)	p(I)	p(Z)	p(IxZ)
CAT (μmol/g)	75.1±6.76	82.5±8.2	57.8±6.76	68.9±6.76	0.045	0.213	0.798
SOD (nmol/mg)	9.55±0.88	8.15±1.08	15.4±0.88	11.2±0.88	<0.001	0.008	0.149
GSH-Px (nmol/mg)	34.4±2.45	35.7±3	30.3±2.45	32.9±2.45	0.200	0.466	0.806

Table 3. Cardiac Protein Expression. SC=sham animals that received standard diet; SZ=sham animals that received diet with zinc supplementation; IC= infarcted animals that received standard diet; IZ= infarcted animals that received diet with zinc supplementation. Nrf-2: nuclear factor erythroid 2-related factor 2; Col I: type I collagen; Col III: type III collagen; IL-10: interleucin 10. *p<0.05 vs. SC, IC and IZ

Variables	SC (n=8)	SZ (n=8)	IC (n=8)	IZ (n=8)	p(I)	p(Z)	p(IxZ)
Nrf-2	0.84±0.14	1.06±0.13	1.05±0,14	1.07±0.14	0.466	0.391	0.465
Col I	0.21±0.05	0.27±0.04	0.32±0.03	0.42±0.04	0.023	0.133	0.675
Col III	1.11±0.18	0.79±0.20	1.18±0.15	1.45±0.16	0.042	0.841	0.091
IL-10	1.32±0.08	1.39±0.09	1.69±0.08	1.53±0.07	0.003	0.606	0.148

in the spleen as well as the amount of total lymphocytes. Zinc increased the amount of CD4+ in infarcted animals, but we did not observe effects of supplementation in relation to Treg cells.

Table 4. Leukocyte Count in the Spleen. SC=sham animals that received standard diet; SZ=sham animals that received diet with zinc supplementation; IC= infarcted animals that received standard diet; IZ= infarcted animals that received diet with zinc supplementation. CD4+CD25+Foxp3: Treg cells. *p<0.05 vs. IC

Variables	SC (n=10)	SZ (n=10)	IC (n=10)	IZ (n=10)	p(I)	p(Z)	p(IxZ)
Lymphocytes	87015± 3033	84506± 8735	88673± 1365	89777± 1222	0.026	0.640	0.233
CD4+	24614± 3146	22937± 5039	22865± 3533	26312± 2793*	0.495	0.458	0.036
CD4+CD25+Foxp3	878± 314	842± 258	1071± 333	1136± 270	0.013	0.877	0.590

As expected, the zinc levels were higher in the supplemented animals (SC=99.1±4.29 µg/dL; SZ=103±3.70 µg/dL; IC=100±4.29 µg/dL; IZ=114±4.29 µg/dL; p(I)=0.145; p(Z)=0.034; p(IxZ)=0.21).

Discussion

The objective of this work was to evaluate the effects of zinc supplementation on cardiac remodeling after myocardial infarction. From the data presented, it can be suggested that variables associated with the remodeling process were attenuated by zinc supplementation.

The role of zinc as a modulator of cardiac changes has been prominent in recent years. Thus, previous studies have examined the cardiac effects of zinc on different clinical scanarios. For instance, a study showed an improvement in the LV relaxation by zinc in prediabetic animals, through the action of this mineral in the proteins linked to calcium transit, resulting in a lower intracellular calcium load [27]. In a study with zinc administration associated with acetylsalicylic acid (ASA) before the ischemic injury by isoproterenol in animals, an improvement in cardiac contractility and lower infiltration of inflammatory cells was observed compared with the group that was previously treated with ASA alone, showing beneficial effects of zinc supplementation in acute myocardial infarction, probably due to the induction of antioxidant enzymes and the anti-inflammatory cytokine TGF-β1 [28]. In humans, serum zinc level was decreased in patients with heart failure and independently predicted clinical status and LV diastolic function [15]. However, the effects of zinc supplementation after infarction were unknown,

The most important result of our study was that zinc supplementation attenuated MI-induced LV remodeling. As expected, the infarction resulted in increased diastolic and systolic diameters of the LV. It should be emphasized that although the remodeling is an extremely complex event, after the infarction this process is clinically recognized by an increase in the LV cavity. In addition, a well-accepted concept is that the remodeling process is usually associated with deterioration of ventricular function [2-5]. Therefore, our study suggests that zinc administration attenuated infarct-induced ventricular remodeling, associated with improved systolic and diastolic function. Interesting, zinc improved some functional variables even in normal rats, similar to the finding of a previous study [8], probably due to a combination of competitive inhibition of calcium influx through the l-type calcium channel, and the modulation of ryanodine receptor-mediated calcium release [8, 10].

The beneficial effects of zinc in our study could be explained by different mechanisms, particularly changes in the extracellular matrix, attenuation of oxidative stress, and modulation of inflammatory response [6-16].

The extracellular matrix has a structural role, supporting cardiomyocytes and vessels of the heart and mediating intercellular communication and metabolic changes [29]. The

main component of the matrix is collagen. The main types of collagen found in cardiac tissue of different species are I and III, which make up 95% of the total collagen. After infarction, there is high accumulation of collagen (fibrosis) on areas of the myocardium. In this context, fibrosis was associated with increased myocardial stiffness, diastolic dysfunction, weakened contraction, impaired coronary flow, and malignant arrhythmias. In addition, fibrosis was a predictor of mortality in patients with cardiac dysfunction [30, 31]. In our study, zinc supplementation did not decrease the accumulation of collagen induced by the infarction. Thus, we could infer that the decrease in fibrosis was not a mechanism involved in the beneficial effects of zinc in this model.

Another potential mechanism involved in the beneficial effects of zinc is oxidative stress. Strong evidence supports an association between cardiac remodeling and oxidative stress resulting from increased reactive species production or decreased antioxidant defense.

Importantly, oxidative stress would lead to several alterations, such as lipid peroxidation, protein oxidation, DNA damage, cellular dysfunction, proliferation of fibroblasts, induction of apoptosis, changes in calcium-transport proteins, and activation of hypertrophy-signaling pathways [32, 33]. Consequently, after infarction, cardiac remodeling was attenuatted by administration of different antioxidants products, including tomato/lycopene [34], rosemary [35], probucol [36], green tea [37], and n-acetylcysteine [38].

As expected, in our study, infarction induced several changes in antioxidant enzymes. Importantly, zinc attenuated the infarct-induced changes. Thus, we conclude that the beneficial effects of zinc in this model were mediated, at least in part, by alteration on redox state, but without participation of Nrf-2.

Inflammation is a key modulator of cardiac remodeling. After myocardial infarction, cardiac repair is initiated by intense inflammation and immune cell infiltration, followed by a reparative phase with scar formation. Persistent inflammatory phase participates in the pathogenesis of adverse ventricular remodeling [39]. Recently, it has been accepted that Treg cell plays a prominent role in this process. Treg cells are identified by the expression of CD4+, CD25+, and fork head box P3 (Foxp3) markers and suppress the early inflammatory phase after infarction, contributing to the beginning of the proliferative/reparative phase, associated with IL-10 levels [40]. Therefore, increased Treg cells protected against adverse remodeling following coronary occlusion in rats, via inhibition of inflammation [41]. In contrast, Treg depletion contributed to early postinfarction dilation by increase in the LV end-diastolic volume [42].

An important issue is that the role of Treg cells in the very late phase after myocardial infarction is unknown. In our study, total lymphocytes and Treg cells were increased in the spleen of infarcted animals, associated with IL-10 levels. Likewise, it is accepted that the spleen acts as a reservatory of leucocytes, and their number in the spleen were several folds higher than in circulation early after myocardial infarction [43]. Therefore, our data suggest that Treg cells might be a modulator of deleterious cardiac remodeling in the chronic phase after myocardial infarction in rats, similar to that occurring in the acute phase. However, zinc administration did not change the Treg cells amount and IL-10 levels.

Conclusion

Zinc supplementation attenuates cardiac remodeling after myocardial infarction in rats; this effect is associated with modulation of antioxidant enzymes, but without the involvement of accumulation of collagens I and III, Nrf-2, IL-10, and Treg cells.

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Disclosure Statement

The authors declare to have no competing interests.

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