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ORIGINAL PAPER

Administration of zinc complex of acetylsalicylic acid after the onset of myocardial injury protects the heart by upregulation of antioxidant enzymes

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Abstract We recently demonstrated that the pre-treatment of rats with zinc and acetylsalicylic acid complex in the form of bis(aspirinato)zinc(II) [$Zn(ASA)_2$] is superior to acetylsalicylic acid in protecting the heart from acute myocardial ischemia. Herein, we hypothesized that $Zn(ASA)_2$ treatment after the onset of an acute myocardial injury could protect the heart. The rats were treated with a vehicle or $Zn(ASA)_2$ after an isoproterenol injection. Isoproterenol-induced cardiac damage [inflammatory infiltration into myocardial tissue, DNA-strand breakage evidenced by TUNEL-assay, increased 11-dehydro thromboxane (TX)B_2-levels, elevated ST-segment, widened QRS complex and prolonged QT-interval] was prevented by the $Zn(ASA)_2$ treatment. In isoproterenol-treated rats, load-independent left ventricular contractility parameters were significantly improved after $Zn(ASA)_2$. Furthermore, $Zn(ASA)_2$ significantly increased the myocardial mRNA-expression of superoxide dismutase-1, glutathione peroxidase-4 and decreased the level of Na^+/K^+/ATPase. Post-conditioning with $Zn(ASA)_2$ protects the heart from acute myocardial ischemia. Its mechanisms of action might involve inhibition of pro-inflammatory prostanoids and upregulation of antioxidant enzymes.

Keywords Myocardial ischemia · Zinc complex of acetylsalicylic acid · Isoproterenol

Introduction

Myocardial ischemic injury is caused by an inadequate supply of oxygen-rich blood and metabolic nutrient requirements to a portion of the heart, due to an increased myocardial substrate demand and/or the narrowing or closure of the coronary arteries [1]. Overproduction of oxygen-derived free radicals, intracellular calcium (Ca^{2+}) overload, adenosine triphosphate (ATP) depletion, acidosis and an increased migration of neutrophils to the ischemic tissue are critical phenomena in the pathogenesis of a myocardial infarction [2]. All of these changes induce myocardial damage, and pharmacological approaches such as antioxidant agents and anti-inflammatory treatments may reduce ischemic injury and facilitate recovery.

Aspirin (acetylsalicylic acid, ASA), a non-steroidal anti-inflammatory drug, is one of the clinically used, long-term medications in the case of ischemic heart disease. In experimental models, it has been shown that its administration significantly protects the heart against catecholamine infusion-induced [3] and epinephrine-induced [4] myocardial necrosis, and against isoproterenol-induced cardiac injury [5]. However, in clinical trials, ASA given to patients soon after the onset of symptoms following myocardial infarction did not prove to be beneficial in reducing the mortality rate [6, 7]. Zinc ions, on the other hand, are known to have an anti-inflammatory characteristic [8] and one of the physiological functions of zinc has been hypothesized to be an endogenous antioxidant [9]. It has been demonstrated that the administration of zinc decreases the magnitude of isoproterenol-induced heart

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injury [10]. Electrocardiographic and ultrastructural studies also showed that zinc protects the heart against catecholamine-induced myocardial changes [11]. Additionally, the cardioprotective effects of zinc in an isolated post-ischemic rat heart model have clearly been demonstrated [12, 13]. This data strongly suggests that zinc might be a useful approach in the prevention of ischemic heart disease. Therefore, a compound of zinc and acetylsalicylic acid was synthesized in the form of bis(aspirinato)zinc(II) complex [($Zn(ASA)_2$, a 2:1 complex of ASA to zinc] [14]. One of its main advantages compared with uncomplexed ASA is that zinc may increase the anti-inflammatory effects of ASA. As a new preventive treatment concept for patients at greater risk of ischemic heart disease, we investigated the effects of $Zn(ASA)_2$ and we recently showed that pre-treatment of rats with $Zn(ASA)_2$ is superior to ASA in preventing electrical, mechanical and histological changes after acute myocardial ischemia [15]. Furthermore, we demonstrated that $Zn(ASA)_2$ reduced restenosis in a rat carotid artery balloon injury model [16].

Isoproterenol (isoprenaline) hydrochloride, a synthetic catecholamine and β1 and β2-adrenergic receptor activator, exerts acute positive inotropic and chronotropic effects on the heart [17]. Its administration in higher doses has been used to induce experimental myocardial infarction in mammals [18]. In certain respects, isoproterenol-induced myocardial alterations are similar to those that occur in human myocardial infarctions. These include changes in serum enzymes [19], electrocardiographic alterations [20], hemodynamic disturbances, Ca^{2+} overload, as well as histological changes [21]. One of the proposed mechanisms to explain the isoproterenol-induced cardiac injury is the excessive production of free radicals and lipid peroxides by catecholamine auto-oxidation.

In some cases, coronary artery disease cannot be treated with percutaneous coronary interventions. For these patients who cannot receive reperfusion therapy, new pharmacological strategies are required. Taking this information into account, we examined whether the administration of $Zn(ASA)_2$ has any protective properties after the onset of an acute myocardial injury induced by isoproterenol in rats. To that end, we evaluated in vivo ventricular mechanical function, assessed biochemical and histological changes, and cardiac gene expression.

Materials and methods

Animals

All experiments were carried out with male Sprague–Dawley rats (250–350 g; Charles River, Sulzfeld, Germany). The animals were housed in a room and under standard laboratory conditions with a 12-h light–dark cycle and were given food and water ad libitum. The rats were acclimatized for at least 1 week before the experiments. Both the care of the animals and the experimental procedures were conducted in accordance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). Our study was approved by the appropriate institutional review committees.

Isoproterenol-induced myocardial ischemia

Isoproterenol was dissolved in distilled water and injected subcutaneously into rats (85 mg/kg, at a volume of 0.1 ml/100 g body weight) daily for 2 consecutive days at an interval of 24 h in order to induce experimental myocardial injury [22].

Experimental groups

The rats were randomly divided into four groups: (1) the control group received 0.5 % methylcellulose solution (vehicle) orally, 30 min after the first injection and 8 h before the second injection of distilled water ($n = 7$); (2) the isoproterenol group received 0.5 % methylcellulose orally, 30 min after the first injection and 8 h before the second injection of isoproterenol ($n = 11$); (3) the isoproterenol plus $Zn(ASA)_2$ group received orally $Zn(ASA)_2$ 30 min after the first injection and 8 h before the second injection of isoproterenol ($n = 8$); and (4) the $Zn(ASA)_2$ rats received $Zn(ASA)_2$ orally, 30 min after the first injection and 8 h before the second injection of distilled water ($n = 7$). The experiment was stopped 17–22 h after the last administration of drugs.

Electrocardiograms and assessment

The rats were anesthetized with sodium pentobarbital (60 mg/kg) intraperitoneally. The body temperature (measured via a rectal probe) was maintained at 37 °C. Standard 12-lead electrocardiograms were recorded using needle electrodes placed subcutaneously. All leads were connected to a standard direct-writing recorder (Mortara Instrument, WI, USA). The paper speed was set at 50 mm/s and the sensitivity at 10 mm/mV. The ECG analysis was evaluated in lead II including the following measurements: ST-segment elevation, QRS complex and QT-intervals. The QT-interval, measured from the onset of the QRS complex to the end of the T wave, was corrected using the normalized Bazett's formula adjusted for rats

[nQTc = QT/(RR/f)1/2] [23]. Electrocardiography was analyzed by an investigator blinded to the experimental groups.

Echocardiography

After ECG recordings, the animals' thoraxes were shaved and transthoracic echocardiography was performed in the supine position by using an HDI 5000 CV echocardiography system (ATL Ultrasound, Philips, Bothell, WA, USA) equipped with a 10-MHz linear probe. Two-dimensional short-axis and long-axis images as well as M-mode recordings at the mid-papillary muscle level were assessed. The study was completed by analyzing the digital images in a blinded fashion using an image analyzing software (HDI Lab, Philips, Bothell, WA, USA). From these images the following parameters were measured: left ventricular anterior wall thickness (AWT), left ventricular posterior wall thickness (PWT) in diastole (index: d) and systole (index: s); left-ventricular end-diastolic (LVEDD) and end-systolic (LVESD) diameters. End-diastolic and end-systolic time points were considered to be those moments when the largest and the smallest left ventricular diameters were detected. All values were calculated as the average from three different cardiac cycles. To estimate the myocardial weight, left-ventricular mass (LV mass) was calculated using the Devereux formula: LV mass (g) = $\{[(\text{LVEDD} + \text{AWTd} + \text{PWTd})^3 - \text{LVEDD}^3] \times 1.04\} \times 0.8 + 0.14$ [24]. Furthermore, left-ventricular volumes were estimated according to the Prolate ellipsoid (length-diameter) formula: $\text{LVEDV} = [(3.14/6) \times \text{LVEDD}^2] \times L_d$, $\text{LVESV} = [(3.14/6) \times \text{LVESD}^2] \times L_s$ [25]. In order to assess cardiac function, fractional shortening (FS) was defined as [(LVEDD − LVESD)/LVEDD] × 100, and ejection fraction (EF) was defined as (SV/LVEDV) × 100.

Hemodynamic studies

The animals were tracheotomised, intubated and artificially ventilated. A polyethylene catheter was inserted into the left external jugular vein for fluid administration purposes. A 2F microtip pressure–volume catheter (SPR-838, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the ascending aorta. After stabilizing for 5 min, the arterial blood pressure was recorded and the catheter was advanced into the left-ventricle under pressure control. With the use of a special pressure–volume analysis program (PVAN, Millar Instruments, Houston, TX, USA), heart rate, systolic blood pressure, diastolic blood pressure, mean arterial pressure, ejection fraction and the time constant of LV pressure decay (Tau; according to the Glanz method [26]) were calculated. LV pressure–volume relations were assessed by transiently compressing the inferior vena cava. The slope E_{max} of the LV end-systolic pressure–volume relationship and preload recruitable stroke work (PRSW) were calculated as load-independent indexes of LV contractility. At the end of each experiment, 0.1 ml of hypertonic saline (5 %) were injected using the central venous line, and using the shift of pressure–volume relations, the parallel conductance volume was calculated with PVAN pressure–volume analysis software (Millar Instruments, Houston, TX, USA). This was used to correct the absolute LV volume. The volume calibration of the conductance system was performed as previously described [26].

Biochemical analysis

Blood was collected in Li-Heparin Monovette®, EDTA, and serum tubes. After centrifugation, plasma and serum samples were obtained. Plasma high sensitive cardiac troponin T and serum zinc levels were determined. Additionally, plasma concentration of 11-dehydro TXB_2, a marker for in vivo TXA_2 synthesis, was measured with a specific immunoassay kit (Cayman Chemical Company, Michigan, USA).

Histological assessment

After the blood samples were collected, the hearts were explanted, pieces of myocardial tissue were fixed in a buffered paraformaldehyde solution (4 %), embedded in paraffin, cut to 5 µm, and stained with hematoxylin and eosin in order to evaluate the degree of inflammation and edema. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed according to the manufacturer's instructions (Chemicon International, Temecula, CA, USA) to detect DNA strand breakages (free 3′-OH DNA ends). The rehydrated sections were digested with 20 µg/ml DNase-free Proteinase K (Sigma-Aldrich, Germany) to retrieve antigenic epitopes, and endogenous peroxidases were blocked with 3 % hydrogen peroxide (H_2O_2). The free 3′-OH termini of the DNA ends were labeled with a reaction mixture of terminal deoxynucleotidyl transferase (TdT) and digoxigenin-dUTP at 37 °C for 1 h (Chemicon International, Temecula, CA, USA). Incorporated digoxigenin-conjugated nucleotides were detected using a horseradish peroxidase conjugated anti-digoxigenin antibody and 3,3′-diaminobenzidine. The sections were counterstained with Gill's hematoxylin. Dehydrated sections were cleared in xylene, mounted with Permount (Fischer Scientific, Germany) and coverslips were applied. Based on the intensity and distribution of the labeling, a semi-quantitative histomorphological assessment was performed using conventional microscopy. For the assessment of TUNEL-labeled

cells, the number of positive cell nuclei/microscopic examination field with 200× magnification was counted in 4 fields for each sample, averaged, and the mean was calculated for each experimental group. The histological evaluation was conducted by an investigator blinded to the experimental groups.

Quantitative real-time polymerase chain reaction (PCR)

After the blood samples were collected, pieces of myocardial tissue were rapidly excised, frozen in liquid nitrogen, and stored at −80 °C. The apex of the hearts, including both infarcted and healthy areas was used for the analysis of gene expression. The total RNA was isolated with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). The RNA concentration and purity were determined photometrically (230, 260 and 280 nm). Reverse transcription was performed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) using 800 ng RNA in a total volume of 20 µl. Quantitative real-time PCR was performed with the Light-Cycler480 system using the LightCycler480 Probes Master and Universal ProbeLibrary probes (Roche, Mannheim, Germany) (Table 1). The conditions for the PCR were as follows: 95 °C for 10 min (1 cycle), 95 °C for 10 s, 60 °C for 30 s (single; 45-cycle quantification), 40 °C for 10 s (1 cycle). The reaction volume was 20 µl. The efficiency of the PCR reaction was confirmed with standard curve analysis. Sample quantifications were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, by using a pool of all cDNAs (positive calibrator). The evaluation was performed with the Light Cycler 480 SW 1.5 software (Roche, Mannheim, Germany).

In vitro organ bath experiments

Isolated thoracic aortic rings from healthy control rats (in each group, 7–8 independent experiments) were mounted on stainless steel hooks under 2 g of resting tension in individual organ baths (Radnoti Glass Technology, Monrovia, CA, USA), containing 25 ml of Krebs–Henseleit solution. The solution was gassed continuously with 95 % O_2 − 5 % CO_2 and warmed to 37 °C. Special attention

Table 1 Sequence of primers for real time PCR and Universal Probe Library (UPL) probes

Gene	Forward (F) and reverse (R) primer	UPL probes
SOD-1	F:5′-GGTCCAGCGGATGAAGAG-3′	5
	R:5′-GGACACATTGGCCACACC-3′	
GPx-4	F:5′-TGGGAAATGCCATCAAATG-3′	25
	R: 5′-CGGCAGGTCCTTCTCTATCA-3′	
Catalase	F:5′-ATCAGGGATGCCATGTTGTT-3′	129
	R: 5′-GGGTCCTTCAGGTGAGTTG-3′	
Cytochrome c oxidase	F:5′-AGCCAAATCTCCCACTTCC-3′	18
	R: 5′-ATAGCTCTCCAAGTGGGATAAGAC-3′	
$Ca_v1.2$, α_{1c}-subunit	F:5′-GAGGGCTGGACAGACGTG-3′	81
	R:5′-TGACCCTATGATGATTAGTGTTACAAA-3′	
SERCA-2	F:5′-ATGGACGAGACGCTCAAGTT-3′	1
	R:5′-GTTTAGGAAGCGGTTACTCCAG-3′	
Calsequestrin	F:5′-ATTTGCGGAGAAGAGTGACC-3′	80
	R: 5′-CCGGGCAACCTGTTTTAG-3′	
Na^+-K^+-ATPase, α_1-subunit	F:5′-CACCAAGATAGTGGAGATTCCTTT-3′	22
	R:5′-TGGGTTCTTGTGAATGGAGA-3′	
Collagen-1	F:5′-CTGGCAACCTCAAGAAGTCC-3′	65
	R: 5′-CAAGTTCCGGTGTGACTCG-3′	
TGF-β_1	F:5′-TCAGACATTCGGGAAGCAGT-3′	56
	R: 5′-ACGCCAGGAATTGTTGCTAT-3′	
β-actin	F:5′-CTAAGGCCAACCGTGAAAAG-3′	115
	R: 5′-TACATGGCTGGGGTGTTGA-3′	
GAPDH	F:5′-CTACCCACGGCAAGTTCAAT-3′	111
	R: 5′-ATTTGATGTTAGCGGGATCG-3′	

SOD-1 Superoxide dismutase-1, *GPx-4* glutathione peroxidase-4, *α1c-subunit; $Ca_v1.2$* L-type calcium-channel, *SERCA-2* sarco(endo)plasmic 2 Ca^{2+}-ATPase-2, *Na^+-K^+-ATPase* sodium–potassium adenosine triphosphatase, *TGF-β_1* transforming growth factor β_1, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase

was paid during the aortic dissection to avoid damaging the endothelium. The tissue was equilibrated for 60 min. During this period, the tension was periodically adjusted to the desired level, and the Krebs–Henseleit solution was changed every 30 min as a precaution against interfering metabolites. Then, the rings were incubated for 30 min with $Zn(ASA)_2$ (10^{-3} M) or with a vehicle (dimethyl sulfoxide, DMSO). The maximal contraction force to potassium chloride (KCl, 80 mM) was determined, and the aortic rings were washed until the resting tension was again obtained. Aortic preparations were then again incubated with $Zn(ASA)_2$ or DMSO then preconstricted with an α-adrenergic receptor agonist, phenylephrine (10^{-9}–10^{-5} M) until a stable plateau was reached. The integrity of the endothelium was verified by assessing the vasorelaxant response of the precontracted rings to the endothelium-dependent vasorelaxant acetylcholine (10^{-9}–10^{-4} M).

Chemicals

$Zn(ASA)_2$ was synthesized and kindly provided by Prof. Hiroyuki Yasui, Kyoto Pharmaceutical University, Japan. For the in vivo experiment, $Zn(ASA)_2$ was suspended in 0.5 % methylcellulose (Caesar and Loretz GmbH, Hilden, Germany) and administered orally at a dose of 100 mg/kg (1 ml/100 g of body weight). For the in vitro organ bath experiments, it was dissolved in DMSO and used at dose of 10^{-3} M. Isoproterenol hydrochloride was purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany).

Statistical analysis

All data is expressed as mean ± standard error of the mean (SEM). Means between groups were compared by 1-way ANOVA followed by an unpaired *t*-test with Bonferroni correction for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Histopathological examination of the cardiac tissues

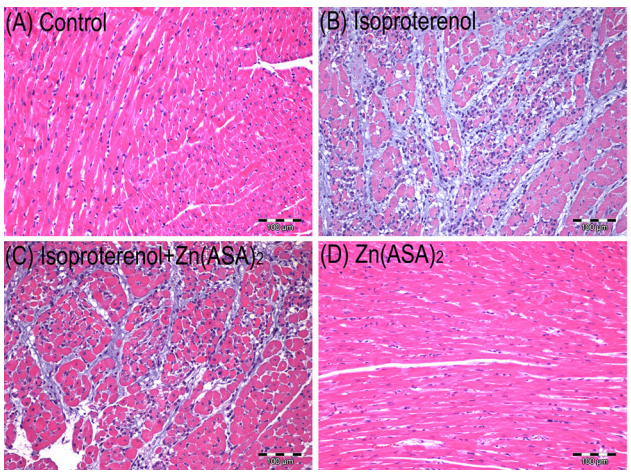
Histological sections of the isoproterenol treated hearts showed myofibrillar degeneration, a dense inflammatory infiltrate and interstitial edema when compared to the control animals (Fig. 1a, b). A treatment with $Zn(ASA)_2$ revealed fewer inflammatory cells in the tissues, as well as less oedema and cardiomyocyte damage (Fig. 1c). Examination of sections from the drug-treated group showed normal architecture (Fig. 1d).

Effects of $Zn(ASA)_2$ post-treatment on ECG

The electrocardiographic pattern is shown in Fig. 2. Rats treated with isoproterenol showed a significant elevation in ST-segments, widened QRS complexes and prolonged corrected QT-intervals in comparison to the control group. Post-treatment with $Zn(ASA)_2$ significantly reversed the changes in the ECG (Table 2).

Effects of $Zn(ASA)_2$ post-treatment on hemodynamic parameters and cardiac function

The changes in the hemodynamic parameters are summarized in Table 3. The ejection fraction values were significantly decreased in isoproterenol injected rats when compared to the control, and similar in the Iso + $Zn(ASA)_2$ and control groups. Additionally, we found that the injection of isoproterenol was associated



Degree of histological changes in myocardium

	Oedema	Inflammation
Control	A	A
Isoproterenol	++	+++
Isoproterenol+$Zn(ASA)_2$	+	++
$Zn(ASA)_2$	A	A

A indicates no changes; ++: moderate changes; +++: marked changes.

Fig. 1 Histopathological examinations of myocardium (hematoxylin and eosin staining). Magnification × 100; *scale bar* = 100 μm. *Iso* isoproterenol, *Zn(ASA)*$_2$ zinc complex of acetylsalicylic acid. *A* indicates no changes; *double plus* moderate changes; *triple plus* marked changes

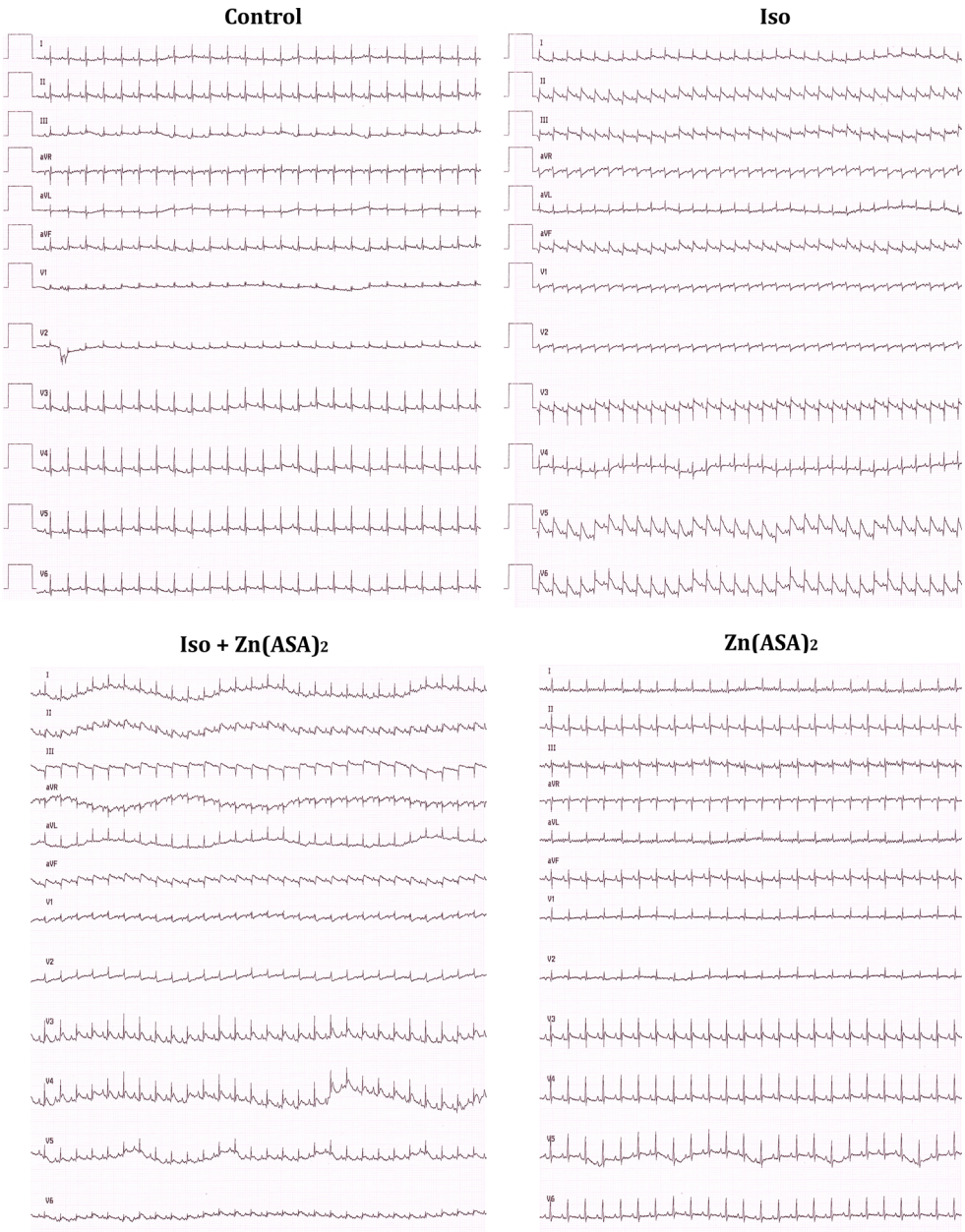


Fig. 2 Representative surface 12-lead ECG tracings. *Iso* indicates isoproterenol and *Zn(ASA)$_2$*, zinc complex of acetylsalicylic acid

Table 2 Electrocardiographic findings

	Control	Iso	Iso + Zn(ASA)$_2$	Zn(ASA)$_2$
ST elevation (mV)	0 ± 0	0.21 ± 0.03*	0.12 ± 0.01*,#	0 ± 0#
QRS (ms)	20 ± 1	33 ± 1*	27 ± 1*,#	22 ± 1*,#
nQTc[a] (ms)	57 ± 2	79 ± 3*	64 ± 1*,#	55 ± 2#

All values are expressed as mean ± SEM

Iso isoproterenol, *Zn(ASA)$_2$* zinc complex of acetylsalicylic acid

* $P < 0.05$ vs control, # $P < 0.05$ vs Iso

[a] nQTc = QT/(RR/f)$^{1/2}$

with significantly decreased systolic performance in comparison to the control group, as reflected by the decreased load-independent contractility index, the slope (E_{max}) of the end-systolic pressure–volume relationship (Table 3). Post-treatment with Zn(ASA)$_2$ resulted in a significant increase in E_{max} and also in PRSW, another relatively load-

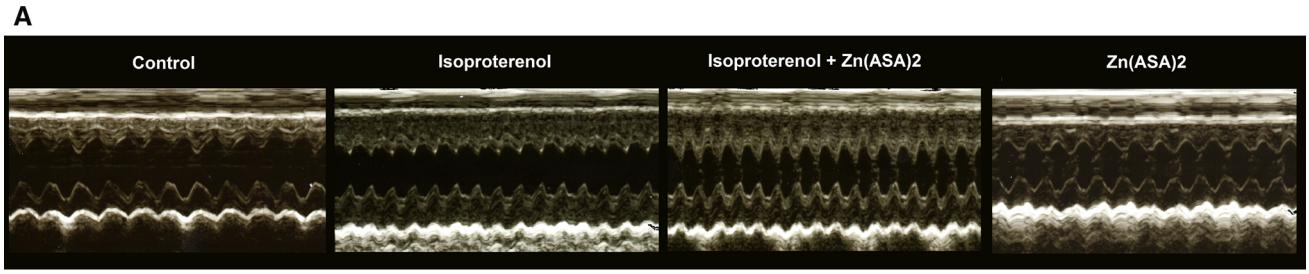
Table 3 Effects of $Zn(ASA)_2$ post-treatment on hemodynamic parameters and left ventricular cardiac function

	Control	Iso	Iso + $Zn(ASA)_2$	$Zn(ASA)_2$
Heart rate (beats/min)	407 ± 10	467 ± 13*	453 ± 18	412 ± 7#
Systolic blood pressure (mmHg)	129 ± 10	107 ± 4	104 ± 3*	126 ± 6
Diastolic blood pressure (mmHg)	101 ± 10	86 ± 3	85 ± 2	99 ± 7
Mean arterial pressure (mmHg)	110 ± 10	93 ± 3	91 ± 2	108 ± 7
Ejection fraction (%)	47 ± 7	27 ± 4*	33 ± 9	55 ± 7#
E_{max} (mmHg/μl)	5 ± 1	3 ± 1*	5 ± 1#	4 ± 0
PRSW (mmHg)	77 ± 7	60 ± 6	85 ± 6#	105 ± 6#
Tau-g	9.2 ± 1.0	14.4 ± 0.9*	13.1 ± 0.6*	10.6 ± 0.5#

All values are expressed as mean ± SEM

Iso isoproterenol, *Zn(ASA)₂* zinc complex of acetylsalicylic acid, E_{max} the slope of the left-ventricular end-systolic pressure–volume relationship, *PRSW* preload recruitable stroke work, *Tau-g* time constant of LV pressure decay

* $P < 0.05$ vs Control; # $P < 0.05$ vs Iso; $ $P < 0.05$ vs Iso + $Zn(ASA)_2$



B

	Control	Isoproterenol	Isoproterenol + $Zn(ASA)_2$	$Zn(ASA)_2$
Heart rate [beats/min]	394 ± 7	514 ± 1*	489 ± 17*	390 ± 20#$
LVESD [mm]	3.53 ± 0.06	3.54 ± 0.27	3.07±0.17	3.16 ± 0.09
LVEDD [mm]	6.31 ± 0.18	5.33 ± 0.23*	5.11±0.31*	5.68 ± 0.16
LVESV [μl]	88 ± 3	107 ± 17	78±9	68 ± 6
LVEDV [μl]	318 ± 21	255 ± 20	229±13*	251 ± 19
Ejection fraction [%]	72 ± 1	59 ± 5*	66±3	73 ± 2#
Fractional shortening [%]	44 ± 1	34 ± 4*	40±2	44 ± 2
LV mass/body weight x 10^{-3}	2.26 ± 0.09	3.95 ± 0.18*	3.93±0.85*	2.33 ± 0.086#$

Fig. 3 Echocardiographic assessment. **a** Representative M-Mode short axis recordings, and **b** echocardiographic parameters. *Zn(ASA)₂* indicates zinc complex of acetylsalicylic acid, *LV* left-ventricular, *LVESD* left-ventricular end-systolic diameter, *LVEDD* left-ventricular end-diastolic diameter, *LVESV* left-ventricular end-systolic volume, *LVEDV* left-ventricular end-diastolic volume. *$P < 0.05$ vs control, #$P < 0.05$ vs isoproterenol, $$P < 0.05$ v. Iso + $Zn(ASA)_2$

independent parameter of cardiac contractility. However, the impaired myocardial relaxation (Tau-g) was not improved by $Zn(ASA)_2$ post-treatment (Table 3).

Effects of $Zn(ASA)_2$ on echocardiographic parameters

In order to study cardiac systolic function, we measured LV ejection fraction and fractional shortening. Our results demonstrate that the ejection fraction and fractional shortening values were significantly decreased in isoproterenol injected rats when compared to the control, and similar in the Iso + $Zn(ASA)_2$ and control groups (Fig. 3). Furthermore, isoproterenol-administered rats had significantly higher echocardiographically measured LV mass as compared to the control animals and treatment of infarcted rats with $Zn(ASA)_2$ did not cause any statistically significant changes. A significant increase in the heart rates values were observed in the isoproterenol and Iso + $Zn(ASA)_2$ groups when compared to the control and $Zn(ASA)_2$ groups. There were no significant differences in the LVESD and LVESV among the studied groups (Fig. 3).

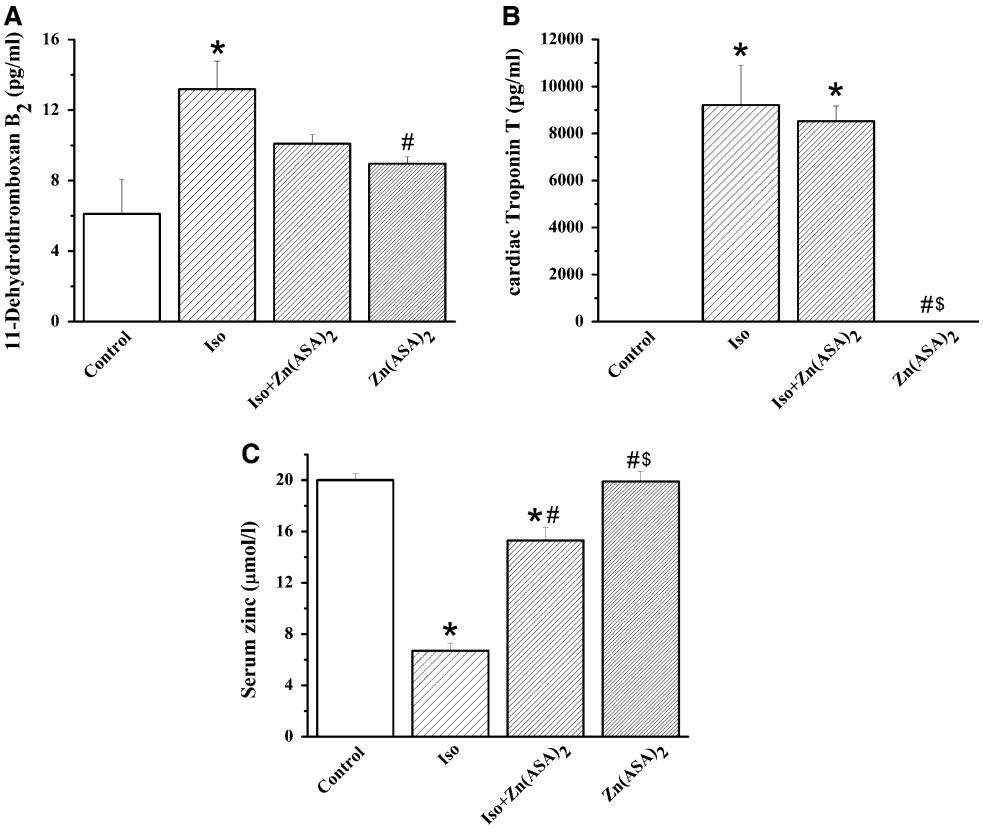


Fig. 4 Effects of zinc complex of acetylsalicylic acid [$Zn(ASA)_2$] on plasma 11-dehydrothromboxane ($TX)B_2$, cardiac troponin T and serum zinc levels in isoproterenol (Iso)-induced myocardial damage. $^{*}P < 0.05$ v. control, $^{\#}P < 0.05$ v. iso, $^{\$}P < 0.05$ v. Iso + $Zn(ASA)_2$

Effects of $Zn(ASA)_2$ post-treatment on plasma 11-dehydro TXB_2, cardiac troponin T and zinc levels

Compared with the control group, the isoproterenol injection resulted in a significant increase in the plasma levels of 11-dehydro TXB_2 (Fig. 4a). Myocardial infarcted rats treated with $Zn(ASA)_2$ showed similar levels of 11-dehydro TXB_2 when compared with the control group. However, the increased plasma levels of high sensitive cardiac troponin-T were not reduced by post-treatment with $Zn(ASA)_2$ (Fig. 4b). Additionally, the serum zinc level in acute myocardial infarcted rats was significantly low as compared to control and $Zn(ASA)_2$ groups. While administration of $Zn(ASA)_2$ alone did not increase the serum zinc content, simultaneous administration of $Zn(ASA)_2$ and isoproterenol resulted in a significant increase of zinc in the serum (Fig. 4c).

Post-treatment with $Zn(ASA)_2$ decreases DNA-strand breaks

Compared with the control, the isoproterenol injection tended to lead the cardiomyocytes into apoptosis ($p = 0.059$), as reflected by the quantitative assessment of TUNEL-positive cell nuclei. Treatment with $Zn(ASA)_2$ significantly decreased isoproterenol-induced DNA-strand breaks (Fig. 5a, b).

Effects of $Zn(ASA)_2$ post-treatment on myocardial gene expression

After acute myocardial infarction, post-treatment with $Zn(ASA)_2$ has no effect on altered myocardial mRNA expression of catalase, cytochrome c oxidase, L-type Ca^2, SERCA, calsequestrin, collagen-I, TGF-β_1 and β-actin (Fig. 6). However, treatment of infarcted rats with $Zn(ASA)_2$ was shown to significantly increase SOD-1, glutathione peroxidase-4 and decrease Na^+/K^+/ATPase mRNA levels (Fig. 6).

In vitro effects of $Zn(ASA)_2$ on contraction of isolated aortic rings

The functional integrity of the endothelium was verified by relaxation of the aortic rings to acetylcholine [maximal endothelium-dependent relaxation (R_{max}) values of the DMSO vehicle, 100 ± 3 % vs $Zn(ASA)_2$, 99 ± 1 %, $P > 0.05$ and pD_2 values of the DMSO vehicle, 7.2 ± 0.2 vs $Zn(ASA)_2$, 7.4 ± 0.4, $P > 0.05$]. Both the maximal contraction and the sensitivity [pD_2 values of the DMSO

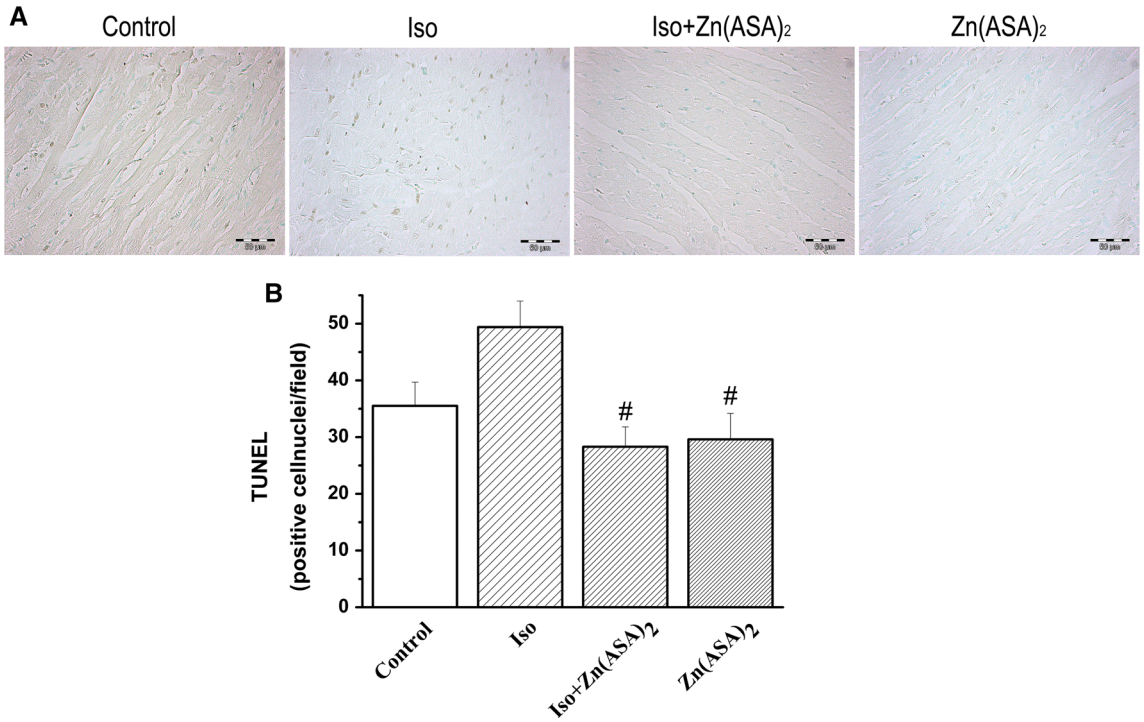


Fig. 5 Zinc complex of acetylsalicylic acid [$Zn(ASA)_2$] decreases DNA-strand breaks. **a** Representative photomicrographs of TUNEL assay (brown staining; magnification ×200; scale bar: 50 μm), and **b** average number of TUNEL-positive cell nuclei in a microscopic field. $^{\#}P < 0.05$ vs iso

vehicle, 6.7 ± 0.1 vs $Zn(ASA)_2$, 7.5 ± 0.1, $P < 0.05$] of the aortic rings to phenylephrine, an α_1-adrenergic agonist, were reduced in the presence of $Zn(ASA)_2$ when compared to the DMSO group (Fig. 7a). However, the drug did not show any antagonistic effect on the contractile responses to high K^+-induced depolarization (Fig. 7b).

Discussion

The aim of this study was to investigate whether the administration of $Zn(ASA)_2$ after the onset of acute myocardial ischemia protects against isoproterenol-induced myocardial injury. We demonstrated that postconditioning with $Zn(ASA)_2$ has cardioprotective effects. Its mechanisms of action implicate the upregulation of the antioxidant enzymes and reduction of vascular tone, in addition to inhibiting pro-inflammatory prostanoids, TXA_2.

Electrocardiographic abnormalities are the main criteria in the diagnosis of myocardial ischemia and infarction [27]. In the present study significant alterations of ECG patterns were observed in myocardial infarcted rats. Significant elevation of the ST-segment after isoproterenol administration is an indicative of sign of myocardial ischemia. Moreover, the prolonged repolarization of the ventricular myocardium following the isoproterenol injection is manifested by an increase in the corrected QT-interval, a predictor for ventricular tachyarrhythmia [28]. Prolonged QRS, which represents a slower than usual depolarization of the ventricles, was observed after the isoproterenol injection. Our results showed that isoproterenol administration exhibited changes in ECG pattern and post-treatment with $Zn(ASA)_2$ prevented the pathological ECG abnormalities.

We and other groups have previously reported that the administration of isoproterenol at high doses causes cardiac dysfunction [22, 29, 30]. In the present work, in order to evaluate the contractile abilities of the heart, different indices were calculated. In our rat model, we found that myocardial ischemia was associated with a significantly decreased ejection fraction using echocardiography and a conductance catheter system. However, the ejection fraction, a widely used clinical parameter of systolic performance, is known to be influenced by both preload and afterload, therefore limiting its usefulness. Myocardial contractility can also be estimated by the slope E_{max} of the ESPVR, as a load-insensitive index of contractility. PRSW, the linear relation between stroke work and end-diastolic volume, is another pressure–volume derived load-independent parameter. We showed that the treatment of myocardial infarcted rats with $Zn(ASA)_2$ improved the LV systolic function. However, $Zn(ASA)_2$ has no effect on the

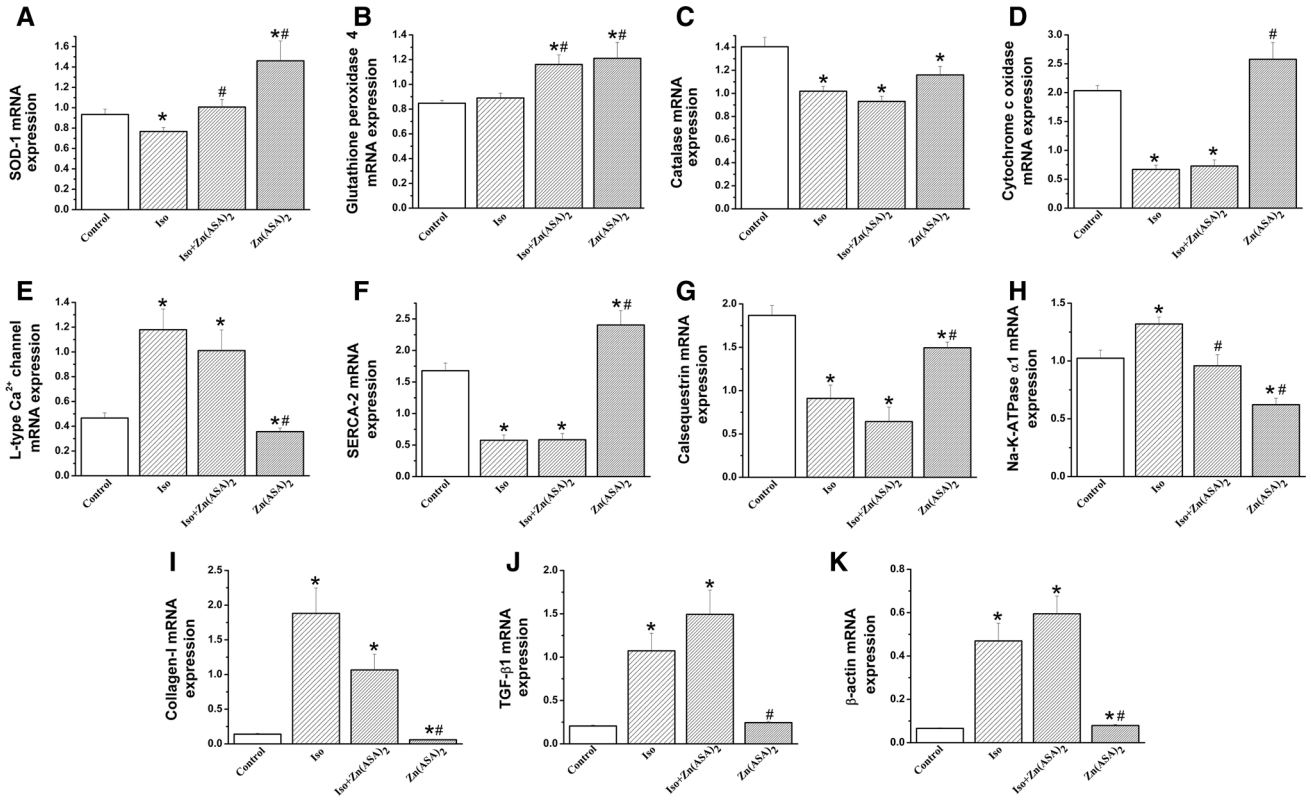


Fig. 6 Effects of zinc complex of acetylsalicylic acid [$Zn(ASA)_2$] on gene expression. Quantitative PCR analysis of myocardial mRNA levels of **a** superoxide dismutase (SOD)-1, **b** glutathione peroxidase 4, **c** catalase, **d** cytochrome c oxidase, **e** L-type calcium channel, **f** sarco(endo)plasmic Ca^{2+}-ATPase (SERCA)-2, **g** calsequestrin, **h** Na^{+}-K^{+}-ATPase, **i** collagen-I, **j** transforming growth factor (TGF)-β1, and **k** β-actin. $^{*}P < 0.05$ vs control, $^{\#}P < 0.05$ vs isoproterenol (iso)

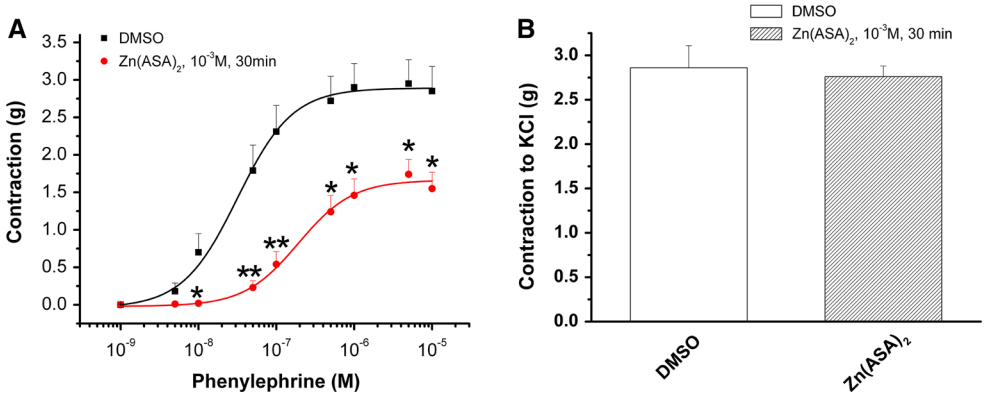


Fig. 7 In vitro effects of zinc complex of acetylsalicylic acid [$Zn(ASA)_2$] on contraction of isolated aortic rings. Contractile responses to **a** α_1-adrenergic receptor agonist phenylephrine, and **b** high K^{+}-induced depolarization. $^{*}P < 0.05$ vs DMSO

diastolic dysfunction (as reflected by prolonged Tau, an index of active LV relaxation). Massive cardiomyocyte death occurs during acute myocardial infarction [31], resulting in the liberation of the intracellular enzymes or markers of irreversible cardiomyocyte injury in the circulation, including cardiac troponin-T, lactate dehydrogenase, and creatine kinase [32, 33]. Furthermore, in the present study, increased LV mass index assessed by echocardiography was observed in the infarcted rats. The increased plasma levels of high sensitive cardiac troponin-T and LV mass were not reduced by $Zn(ASA)_2$ administration despite hemodynamic improvement. Our observations support the view that $Zn(ASA)_2$ had no protective effect on irreversible cardiomyocyte injury but may

partially rescue cardiomyocytes from border zones and remote areas of myocardial infarction, improving their function which in turn leads to an improved global cardiac performance.

Estimation of serum zinc levels can be diagnostic of various diseases and it has been shown that zinc levels fell following myocardial infarction and myocardial ischemia [34, 35]. This might be an indicator for either increased consumption or loss in injured tissue. In the present study, we demonstrated a significant increase of serum zinc after oral administration of $Zn(ASA)_2$, which might correlate with protection against isoproterenol-induced myocardial infarction. In line with our observations, it has been recently shown that a fall in serum zinc is a useful diagnostic test for acute myocardial infarction and oral zinc administration to the patients of acute MI may be helpful in the prognosis [36].

It was reported that oxidative stress was the main mechanism of isoproterenol-induced myocardial necrosis [37]. Antioxidant enzymes such as SOD-1, glutathione peroxidase and catalase provide the first line of cellular defense against harmful oxidative stress. In the present study, we reported that the mRNA levels of SOD-1 and glutathione peroxidase-4 were significantly increased by $Zn(ASA)_2$, indicating that the induction of genes encoding the antioxidant enzymes during oxidative stress might be an important component in mediating the cardioprotective effect of $Zn(ASA)_2$. On a molecular level, oxidative stress includes lipid peroxidation [38], protein oxidation [39], and DNA oxidative damage [40]. One of the most widely used methods for detecting DNA breaks and DNA fragmentation in tissue sections is TUNEL, which was described to detect cells undergoing apoptosis [41]. In the process of tissue damage during myocardial infarction, most cells in the damaged area die by necrosis, but oxidative stress-induced cell death, such as apoptosis, also plays an important role [42]. In the present study, upon treatment of the myocardial infarcted rats with $Zn(ASA)_2$, the number of cardiac apoptotic nuclei was significantly decreased.

The production of TXA_2, a metabolite of arachidonic acid, increases during myocardial ischemia, contributing to tissue damage [43, 44]. The activation of platelet aggregation and its release, vasoconstriction, as well as augmentation of polymorphonuclear cell adhesiveness by TXA_2 [45] are thought to contribute to the pathophysiology of acute myocardial ischemia. It is not surprising that in the present study, the treatment of infarcted rats with $Zn(ASA)_2$ significantly decreased plasma concentrations of 11-dehydro TXB_2, a metabolite of the vasoconstrictor agent TXA_2. This can be attributed to the inhibition of pro-inflammatory factors by the $Zn(ASA)_2$ complex. The histopathological examination of myocardial tissues supports the idea that $Zn(ASA)_2$ also seems to reduce inflammation and leukocyte migration. In addition, our in vitro experiments on isolated aortic rings show that $Zn(ASA)_2$ decreases the contractile response to phenylephrine, an agonist of the α_1-adrenergic receptor. Taking these facts into account, our data further confirms the cardioprotective effect of oral administration of $Zn(ASA)_2$ in rats, which can also be explained in part by its effect on the vascular tone. β-actin, a cytoarchitectural protein, is upregulated in myocardial remodelling, in response to either pressure or volume overload [46]. In our study, an increased β-actin mRNA expression after the isoproterenol injection may characterize the early response of the myocardium to hypertrophic stimuli. However, in the present set-up, $Zn(ASA)_2$ had no significant influence on β-actin.

Conclusions

We demonstrated that the oral administration of $Zn(ASA)_2$, after the onset of acute myocardial ischemia, protects the heart. Its mechanisms of action implicate the upregulation of antioxidant enzymes and reduction of vascular tone, in addition to inhibiting pro-inflammatory prostanoid TXA_2. The effects of $Zn(ASA)_2$ must be evaluated in experimental large animal models of myocardial infarction, which are more representative of clinical conditions, to explore other mechanisms. We nevertheless hypothesize that the use of $Zn(ASA)_2$ may be a new treatment option for coronary artery disease.

Study limitations

Isoproterenol, if administered in suprapharmacological doses is a well-known inducer of myocardial ischemia [47] and widely used as a model of assessing cardioprotective agents [48]. Although this rat model does not exactly reflect the clinical situation in terms of coronary occlusion and regional myocardial infarction, administration of isoproterenol has been reported to produce “infarct-like” damage in experimental animals. Another limitation of our study was that we did not assess protein expression, but confined the investigation to the mRNA patterns. Furthermore, to get a better insight into the mechanisms of the beneficial effects of $Zn(ASA)_2$ on cardiac function in our rat model, we investigated the possible protective effects of the drug on vascular function by isometric contractile force measurements in the thoracic aorta. We performed experiments with phenylephrine-induced contraction on isolated aortic rings while phenylephrine imitates the action of neurotransmitters such as adrenaline or norepinephrine and is commonly used to contract the blood vessels. Coronary arteries in the rat heart could not be used due to their small size in our setting, and difference in anatomical structure. Due to the

difference in anatomical structure between rat and human coronary arteries, the findings of our study cannot be directly applied to the clinical situation. Future work should examine a deep mechanistic understanding on molecular levels of the $Zn(ASA)_2$-induced cardioprotection.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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