Original Communication

Combined 1,25-Dihydroxyvitamin D and Resveratrol: A Novel Therapeutic Approach to Ameliorate Ischemia Reperfusion-Induced Myocardial Injury

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Abstract: The aim of this study was to assess the effect of combined 1,25-dihydroxyvitamin D (1,25 D) and resveratrol on cardiac arrhythmias, infarct size, and transcription of catalase, thioredoxin-1 and B-cell lymphoma 2 (Bcl-2), following myocardial ischemia-reperfusion (IR) in male rats. Ligation of coronary artery was performed in rats (n = 6 per group) without any treatment (IR group), pretreated with 0.1 μg/kg/day of 1,25 D (1,25 D + IR), 1 mg/kg/day of resveratrol (Res + IR) or a combination (1,25 D + Res + IR) for 14 days. Arrhythmias were analyzed according to the Lambeth conventions, and infarct size was measured by 2,3,5-triphenyl-2H-tetrazolium chloride staining. Expression of prosurvival genes was evaluated by real-time polymerase chain reaction. In the 1,25 D + Res + IR group the mean infarct size was 17.6 ± 3.5 %, which was significantly less than that in the IR, 1,25 D + IR, and Res + IR groups ($p < 0.001$). Although the single therapy of either 1,25 D or resveratrol did not change the incidence of arrhythmias significantly, a reduction in the number of ventricular ectopic beats was noted in group 1,25 D + Res + IR (179.19 ± 58.87, $p < 0.001$ vs IR; $p < 0.05$ vs Res + IR; $p < 0.01$ vs Vit D + IR). Combination of 1,25 D and resveratrol increased transcription of catalase by 119 ± 37 % ($p < 0.001$ vs IR, $p < 0.01$ vs Res + IR, $p < 0.001$ vs 1,25 D + IR). Our study showed that combination of a non-hypotensive dose of 1,25 D and resveratrol can be a novel and effective strategy for protecting against ischemia.

Key words: 1,25-Dihydroxyvitamin D, resveratrol, myocardial ischemia-reperfusion, catalase

Introduction

Epidemiologic studies have shown the association between vitamin D deficiency and etiology and pathogenesis of cardiovascular diseases including hypertension, myocardial infarction and congestive heart failure [1–3]. Experimental works on cardioprotective effects of vitamin D in animal as well as cellular models of cardiovascular diseases have provided us with invaluable information in recent years. It has been shown that vitamin D deficiency or ablation of vitamin D receptor (VDR) induces marked changes in morphology and function of the myocardium, characterized by hypertrophy and fibrosis [4, 5]. Treatment of Dahl salt-sensitive mice that were fed a high-salt diet, with vitamin D or its analog paricalcitol, will markedly reduce anatomical, functional, biochemical, and molecular changes indicative of cardiac hypertrophy and dysfunction [6, 7]. Administration of paricalcitol in mice with heart failure after myocardial infarction also reduced apoptosis, inflammation, and fibrosis in the myocardium [8].

Some studies have demonstrated the presence of VDR in cardiomyocytes [5, 9], vascular smooth muscle cells [10], and endothelial cells [11], and have indicated that vitamin D directly affects cellular and molecular signaling pathways in cardiomyocytes, although the exact mechanisms are still unknown.

Sirtuins (sirt 1 – sirt 7) are a group of regulatory molecules that have recently attracted the attention of cardiovascular researchers. They are involved in some important cellular processes such as differentiation, apoptosis, proliferation, and metabolism in different tissues including heart [12, 13]. Sirt 1 is a histone deacetylase class II expressed in cardiomyocytes, it regulates cellular processes related to growth, apoptosis, and survival through deacetylation of a large array of non-histone proteins [such as Rab, p53, Foxo, Bcl-6 (B-cell lymphoma 6), PGC(peroxisome proliferator-activated receptor gamma coactivator)-Tx and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)] and histones (H1, H3, H4) [14, 15]. Sirt 1 deacetylates the forkhead winged helix family of transcription factors, known as the forkhead box (Fox), by which synthesis of anti-oxidants such as Mn-SOD (superoxide dismutase) and catalase as well as anti-apoptotic factors such as Bcl-2, PINK1 (PTEN-induced putative kinase 1) and CITED2 (Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2) are increased, but inhibits the pro-apoptotic foxo target genes BIM (BCL2 L11, protein from Bcl-2 protein family) and FAS (FAS ligand receptor), thereby promoting cellular resistance against oxidative stress. Therefore, low-to-moderate increase of these proteins in the heart can protect the heart against hypertrophy, apoptosis, and cardiac dysfunction [16, 17].

Resveratrol (3,5,4′-trihydroxy-trans stilbene), a herbal phenolic phytoalexin, is known as an activator of sirt 1. Studies have shown that resveratrol can prevent myocardial hypertrophy in both isolated murine cardiomyocytes and rats under abdominal aortic stenosis experiments [18–20]. On the other hand, resveratrol increases the levels of mRNA and protein of anti-oxidant enzyme Mn-SOD and also the prosurvival factor thioredoxin in cardiomyocytes, thereby improving cardiac function [21–23].

The probable relationship between vitamin D and sirtuins has been shown in a few studies. Guo et al. indicated that resveratrol in combination with 1,25-dihydroxyvitamin D3 (1,25 D) can improve immune system function [24]. Activation of sirt 1 is mentioned as a mechanism for anti-oxidant effects of vitamin D in endothelial cells [25]. Cancer chemoprevention by this vitamin is also claimed [26]. Epigenetic modification of the vitamin D receptor by histone deacetylases including sirtuins is shown, too [27].

Despite the importance of vitamin D and resveratrol in cardiovascular health, there is no report on probable synergistic effects of these two factors in cardioprotection against myocardial ischemia-reperfusion injury. Thus, it seems that studies on putative synergistic effects of these two factors would be interesting and novel. Therefore, the aim of this study was to assess the effect of combined 1,25 D and resveratrol on cardiac arrhythmias and infarct size following acute myocardial ischemia-reperfusion in rats. The level of transcription of prosurvival factors including catalase, thioredoxin I and Bcl-2 was evaluated, too.

Materials and methods

Male Wistar rats weighing 250–300 g were maintained in a temperature-controlled room on a 12-h dark / 12-h light cycle. They had access to water and standard food. Ethical guidelines concerning work on animals, approved by the Shahid Sadoughi University of medical sciences in Yazd, Iran, were followed (approval letter No. 2378).

Experimental groups

We had two sets of experiments. In the first step, we aimed at the evaluation of effects of intraperitoneal

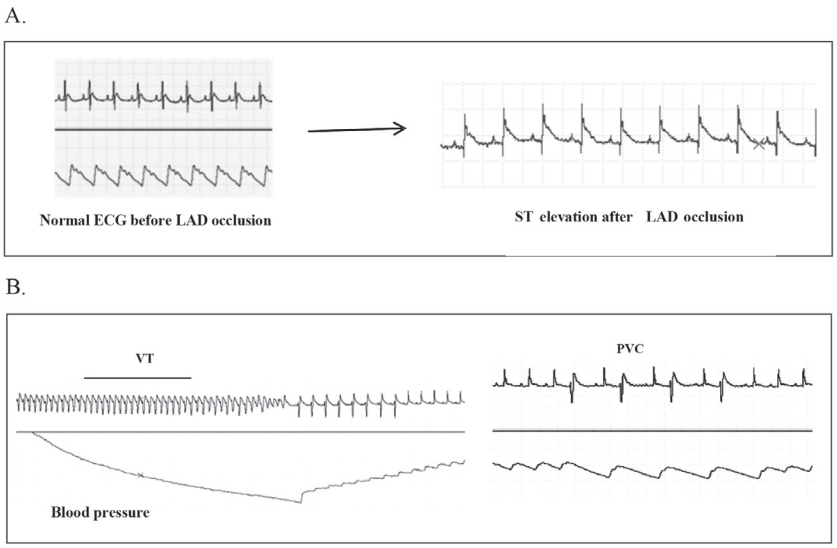

Figure 1: ST-segment elevation is the most important indicator of left anterior descending (LAD) branch of coronary artery occlusion (A). At the first 10 min of reperfusion, many arrhythmias appear in the electrocardiogram (ECG), such as ventricular tachycardia (VT) and premature ventricular contraction (PVC) (B).

injection of 1,25 D and resveratrol on arrhythmias induced by ischemia-reperfusion and on the extent of infarct size; so the animals were randomly assigned to one of the following groups (n = 10 in each group): 1) ischemia-reperfusion (IR): undergoing timed ligation of the left anterior descending (LAD) branch of the coronary artery, without any other intervention; 2) 1,25 D + IR: receiving 0.1 μg/kg/day 1,25-dihydroxyvitamin D (Daru-pakhsh, Iran) for 14 days before IR; 3) Pro + IR: were given propylene glycol (Sigma-Aldrich, USA) as a solvent for vitamin D before IR; 4) Res + IR: 1 mg/kg/day resveratrol (Sigma-Aldrich, USA) for 14 days before IR; 5) DMSO + IR: dimethyl sulfoxide (DMSO) as the solvent of resveratrol; and 6) group 1,25 D + Res + IR: received both 1,25 D and resveratrol before IR. In all experimental groups the drugs were injected intraperitoneally.

In the second experiment, expression of genes for catalase, thioredoxin I, and Bcl 2 in response to resveratrol and 1,25 D was evaluated. For this purpose another 6 groups of rats were studied (n = 6). At the end of reperfusion, the LAD was re-occluded and Evans Blue injected. The pale area (ischemic part) was transferred to liquid nitrogen and then frozen at -80 °C. Since arrhythmias were recorded in both groups of rats (i. e., those targeted at measurement of infarct size, n = 10, and those for gene expression analysis, n = 6), the data related to arrhythmias are evaluated at n = 16.

Our previous pilot study on rats showed that the dose of 0.01 μg/kg of 1,25 D 3×/week for 3 months had no cardioprotective effect against IR injury, either used alone or in combination with losartan or resveratrol. The dose 1 μg/kg/day for 1 month resulted in unexplained severe weight loss and changes in blood pressure (we are investigating this effect). So, the non-calcemic dose of 0.1 μg/kg/day was chosen for this study because it does not affect hemodynamic parameters.

Measurement of serum vitamin D and calcium

To ensure that serum concentration of calcium will not change significantly in the group that received 1,25 D, we obtained a serum sample from each animal at the end of reperfusion, and measured calcium by a spectrophotometer (model Clinic II, Tajhizat Sanjesh, Iran) using a certified kit (Darmankav, Iran).

IR induction model

Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal), and intubated before being ventilated artificially with room air at a frequency of 80 inflations/min on a tidal volume of 1 mL/100 g. Lead II of electrocardiogram (ECG) was recorded via cutaneous needle electrodes. Arterial blood pressure in the left carotid artery was under continuous monitoring by a blood pressure transducer. A left thoracotomy in the fourth intercostal space provided access to the heart, the pericardium was incised, and the heart exposed. In the IR group, occlusion of the LAD branch was maintained for 30 min by lifting a 5/0 silk thread passing below the LAD through a piece of polyethylene

tube. Criteria for confirmation of successful coronary occlusion were appearance of a pale color in the distal myocardium, ST elevation on ECG (Figure 1 A), and a fall in blood pressure. In all IR groups, we allowed reperfusion of ischemic myocardium for 120 min after 30 min of ischemia by loosening the silk thread.

Evaluation of IR-induced arrhythmias

In the induced ischemia model, ligation of the coronary artery causes ST elevation, and arrhythmias are usually not frequent during ischemia, but appear after reperfusion. In Figure 1 some examples of arrhythmias are shown. Arrhythmias were assessed according to Lambeth diagnostic criteria [28]. Each recognizable and isolated premature QRS complex was regarded a premature ventricular beat (PVB). PVB also included any case of bigeminy or Salvos arrhythmia. Each 4 or more successive PVB were considered a ventricular tachycardia (VT) episode. When the QRS complexes were not discernible from each other and the heart rate was not enumerable, we regarded it as ventricular fibrillation (VF).

Infarct size measurement

At the end of the reperfusion period, the LAD was again occluded, and 1 mL of 1 % Evans Blue dye injected into the atria to mark the non-ischemic area blue and the ischemic part (known as area at risk, AAR) pale. The heart was removed, placed in a matrix and stored at -20 °C to be later cut into 2-mm slices from apex to base. The slices were incubated in 1 % 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) solution at 37 °C for 20 min. TTC reacts with NADH dehydrogenase in living tissues and produces a color. Dead cells are not stained due to leaking of this enzyme through ruptured cell membranes. Infarcted areas become pale, but viable portions turn red. Both sides of sections were scanned, and the ischemic areas as well as infracted areas on both sides were measured by image analysis software (ImageJ) and their averages determined. Infarct size was recorded as percentage of ischemic area.

Real-time polymerase chain reaction (RT-PCR) analysis of mRNA level

For evaluation of changes in transcription of genes, total RNA extraction was performed by the RNeasy fibrous tissue mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Concentrations of RNA were determined by measuring its absorbance at 260 nm, and purity of the product was assessed by 260/280 nm absorbance ratio by NanoDrop spectrophotometer (model 2000, Thermo Fisher Scientific, Waltham, MA, USA). Synthesis of first-strand cDNA was done using 1 μg of total RNA with random hexamers, dNTP and Moloney murine leukemia virus reverse transcriptase (Fermentas, Hanover, MD, USA), at a total volume of 20 μL. RT-PCR was performed using the Rotor Gene system (Qiagen) and SYBR Green I. Relative quantity of gene expression was analyzed according to the Pfaffl method. B-actin was used to normalize target genes expression. Nucleotide sequences of the primer sets used for RT-PCR are shown in Table I.

Statistical analysis

Hemodynamic parameters within each group were analyzed by paired t-test. Cardiac arrhythmias, hemodynamic parameters and infarct size were compared between groups by Kruskal-Wallis test with Dunn's

Table I: Primer sequences for RT-PCR of the candidate genes.

Gene	Primer sequences
B-actin	F: 5′-AACCCTAAGGCCAACCGTGAAAAGAT-3′ R: 5′-ACCGCTCGTTGCCAATAGTGATG-3′
Trx-1	F: 5′-TTCCTTGAAGTAGACGTGGATGAC-3′ R: 5′-AGAGAACTCCCCAACCTTTTGAC-3′
Bcl-2	F: 5′-GATTGTGGCCTTCTTTGAGT-3′ R: 5′-ATAGTTCCACAA AGGCATCC-3′
Catalase	F: 5′-CTGACGTCCACCCTGACT -3′ R: 5′-GGCAGCTATGTGAGAGCC-3′

Trx-1: thioredoxin-1. Bcl-2: b-cell lymphoma-2.

Table II: Hemodynamic parameters*. Changes in blood pressure (BP) and heart rate (HR) at the baseline phase (before LAD occlusion), 5 min after the left anterior descending branch of coronary artery (LAD) occlusion, and at the end of reperfusion in groups ischemia-reperfusion (IR), 1,25 D, propylene glycol (Pro), resveratrol (Res), and DMSO.

Groups	Baseline		After LAD occlusion		End of reperfusion	
	BP	HR	BP	HR	BP	HR
IR	118 ± 8.9	374 ± 21	80.1 ± 10.4*	401 ± 33	106 ± 11.5	388 ± 31
1,25 D + IR	113.7 ± 12.4	369 ± 19	82.4 ± 15.3*	396 ± 27	97.8 ± 13.4	391 ± 24
Pro + IR	121 ± 11.3	391 ± 25	96.2 ± 13.7**	411 ± 31	113.4 ± 18.2	403 ± 26
Res + IR	116.5 ± 14.2	386 ± 31	81.3 ± 17.1*	407 ± 41	109 ± 20.5	371 ± 34
DMSO + IR	109 ± 17.7	366 ± 38	77.8 ± 20.3*	387 ± 39	117.6 ± 19.8	375 ± 42
1,25 D + Res + IR	112.1 ± 10.9	39 ± 26	93.6 ± 16.16*	422 ± 25	107.6 ± 15.9	415 ± 30

*Data are shown as mean ± SD. *: $p < 0.05$; **: $p < 0.01$ in comparison with the baseline condition in the same group (n = 16).

multiple comparison post-test. Ventricular fibrillation incidences were compared between groups by Fisher's exact test. Multiple comparisons were done for changes in the level of mRNA by one-way ANOVA. Due to significant differences in overall ANOVA, a comparison between groups was performed by Tukey's comparison test. Data are shown as mean ± SD. $P < 0.05$ is considered as the level of significance.

Results

Changes in serum level of calcium as a response to 1,25-dihydroxyvitamin D

Measurement of serum calcium in the IR and 1,25 D + IR groups indicated that the serum level of calcium in the latter group is 9.01 ± 1.1 mg/dL, being relatively equal to that of the IR group (9.04 ± 0.71).

Hemodynamic parameters

After general anesthesia and use of ventilator, the rat carotid artery was cannulated and its indwelling catheter connected to the pressure transducer. Electrocardiography leads were attached to the limbs to continuously monitor the blood pressure and ECG throughout the experiment. Blood pressure and heart rate of the animals before LAD occlusion and ischemia induction were regarded as baseline values. Immediately after occlusion, there was a sudden drop in blood pressure, which lasted for 10–15 min before rising up to a level mildly below normal, which persisted to the end of the ischemia period. Table II shows changes in blood pressure and heart rate of various groups before LAD occlusion, 5 min after LAD occlusion, and at cessation of reperfusion.

A noticeable point is lack of significant difference in blood pressure and heart rate between experimental groups at baseline level. It means that administration of 1,25 D and resveratrol, either alone or combined, had no effect on blood pressure and

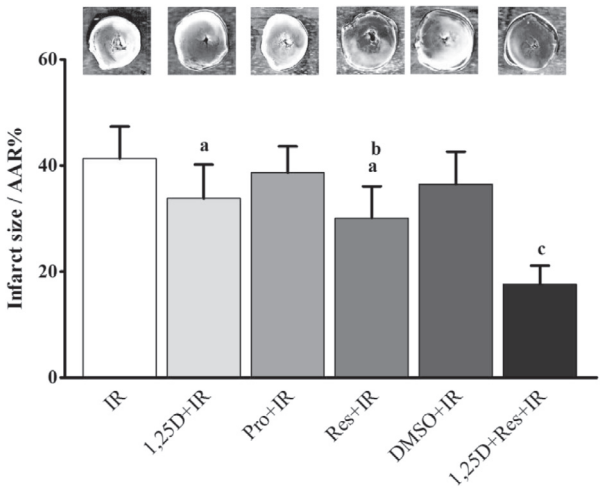

Figure 2: Comparison of the infarct size/area at risk by TTC staining in rats subjected to ischemia reperfusion (IR) without any treatment and in rats treated with 1,25-dihydroxyvitamin D (1,25 D), propylene glycol (Pro), resveratrol (Res), and DMSO. The white regions represent the infracted area. Data are represented as mean ± SD (n = 10). a: vs 1,25 D + Res + IR with $p < 0.001$, b: vs IR with $p < 0.05$. c: vs IR with $p < 0.001$.

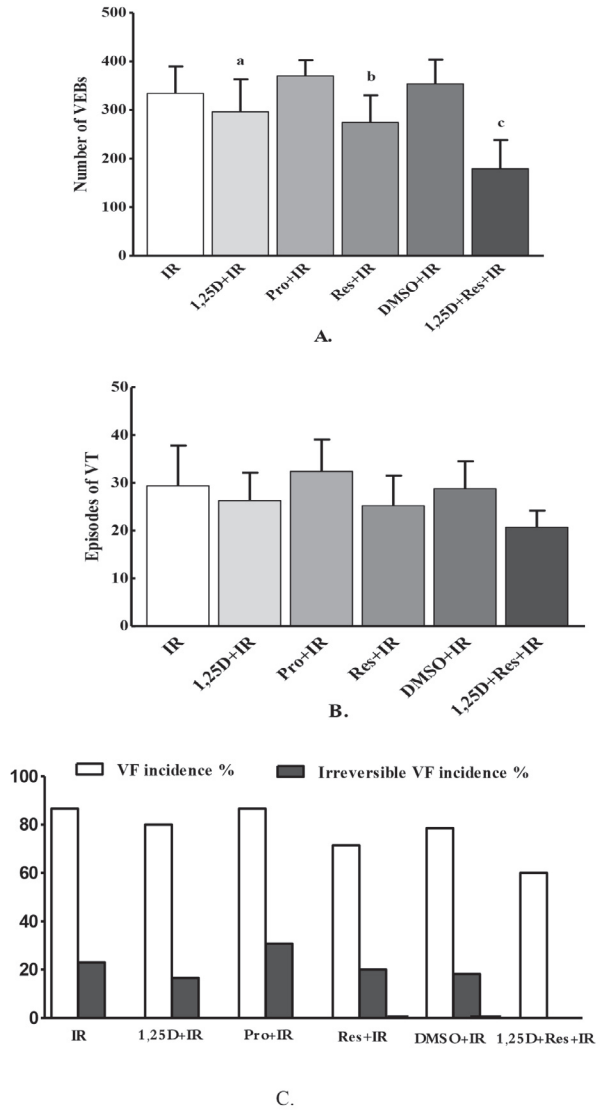

Figure 3: Frequency of arrhythmias of reperfusion period as a response to 1,25-dihydroxyvitamin D (1,25 D) and resveratrol (Res). A, total count of ventricular ectopic beats (VEBs); B, episodes of ventricular tachycardia (VT); in A and B parts data are represented as mean ± SD. C, incidence of reversible and irreversible ventricular fibrillation (VF) (n = 16). a: vs 1,25 D + Res + IR with p <0.01. b: vs 1,25 D + Res + IR with p <0.05. c: vs IR with p <0.001.

heart rate. Also, there was no significant difference in blood pressure and heart rate between groups at the end of reperfusion, which has been the sampling time for evaluation of infarct size and change in expression of genes.

The effects of 1,25-dihydroxyvitamin D and resveratrol on infarct size

Examination of the infarct size through staining with TTC showed that the size of infarction in the IR group is 41.3 ± 6.1 % when compared with the ischemic area, but in the group 1,25 D ± IR it is 33.8 ± 6.4 %, which is not significantly less than in the IR group. In the Res ± IR group the infarct size decreased to 30.1 ± 5.9 %, which is significantly less than the IR group ($p < 0.01$). Simultaneous administration of 1,25 D and resveratrol in the 1,25 D ± Res + IR group resulted in dramatic reduction of infarct size to 17.6 ± 3.5 %, which shows a significant change in comparison with groups IR, 1,25 D + IR, and Res + IR ($p < 0.001$, Fig. 2).

The effects of 1,25 D and resveratrol on frequency of reperfusion arrhythmias

One of the measures for arrhythmias is enumeration of total ventricular ectopic beats (VEBs) during the ischemia-reperfusion period. In the ischemia-reperfusion model performed in our lab, the number of arrhythmias during ischemia is negligible, but there are numerous arrhythmias after initiation of reperfusion, which mostly continue for 10 min but decrease thereafter (Fig. 1). So, the number of arrhythmias belongs to the reperfusion period. As is seen in Figure 3 A, the number of VEBs in the 1,25 D group and the resveratrol group did not decrease significantly in comparison to the IR group (1,25 D + IR: 296.1 ± 66.9, Res + IR: 274.3 ± 55.7 vs IR: 334.2 ± 55.1). However, simultaneous use of 1,25 D and resveratrol decreased VEBs to a significant degree (179.2 ± 58.9, $p < 0.001$ vs IR; $p < 0.05$ vs Res + IR; $p < 0.01$ vs 1,25 D + IR).

Another measure of ventricular arrhythmias is the count of ventricular tachycardia (VT) episodes according to the Lambeth convention. As is demonstrated in Figure 2B, the number of VT episodes in none of the experimental groups was significantly lower than in the IR group.

One of the common and certainly fatal arrhythmias due to ischemia-reperfusion is ventricular fibrillation (VF). Some of the VF episodes can be irreversible, leading to death. The percentage of irreversible VF may also be used as a measure for the evaluation of protective effects of a drug. In Figure 2C the percentage of irreversible VF in each group is shown as a fraction of total VF episodes, which displays the fact that although the frequency of irreversible VF is zero in

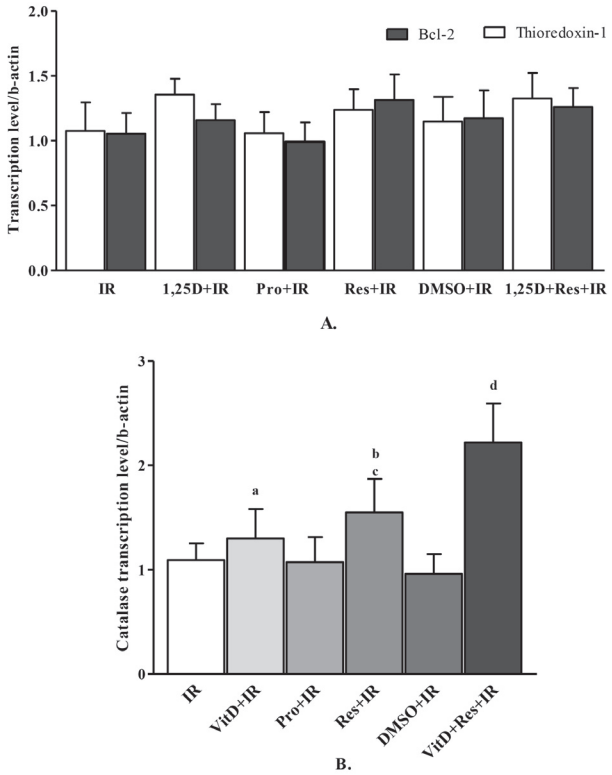

Figure 4: Transcription of survival proteins, including Trx-1, Bcl-2 (A) and catalase (B), in the groups ischemia-reperfusion (IR), 1,25-dihydroxyvitamin D (1,25 D), propylene glycol (Pro), resveratrol (Res) and DMSO. Data are presented as mean ± SD (n = 6). a: vs 1,25 D + Res + IR with $p < 0.001$. b: vs 1,25 D + Res + IR with $p < 0.01$. c: vs IR with $p < 0.05$. d: vs IR with $p < 0.001$.

the group 1,25 D + Res + IR, there is no significant difference among the experimental groups statistically.

Effects of 1,25 D and resveratrol on transcription of survival factors

Results of assessment of transcription level of genes related to survival proteins including Trx-1, catalase and Bcl-2 in ischemic hearts are shown in Figure 4. It was found that administration of 1,25 D alone or in combination with resveratrol did not increase Trx-1 and Bcl-2 mRNA levels in ischemic hearts significantly.

As is seen in Figure 4C, resveratrol alone increased the amount of catalase transcription level in ischemic hearts as compared to the IR group (55.3 ± 32 %, $p < 0.05$), but elevated catalase mRNA in the 1,25 D + IR group was not significantly different. Simultaneous use of 1,25 D and resveratrol increased the level of transcription of the catalase gene to a significant degree (122 ± 37 %) as compared to the IR ($p < 0.001$), 1,25 D + IR ($p < 0.001$) and Res + IR groups ($p < 0.01$).

Discussion

This study showed that co-administration of 1,25 dihydroxyvitamin D and resveratrol can decrease infarct size and chance of arrhythmia in hearts of rats exposed to ischemia-reperfusion, and this decrease is associated with upregulation of catalase. In other words, the combination of 1,25 D and resveratrol has a therapeutic advantage toward amelioration of the injuries resulting from myocardial IR.

Vitamin D and resveratrol are currently interesting molecules in cardiovascular studies. Although there has been plenty of work devoted to cardiovascular effects of each them individually, no study has addressed probable common activities or possible interaction of these two factors. Hayes pointed out some similarities and possible interactions between vitamin D and resveratrol in his review, and mentioned the hormesis phenomenon, anti-oxidant actions, and effect on programmed cell death as the shared biologic processes between them [29]. A few studies were done on the relationship between vitamin D and sirtuins, with interesting results. Combined use of resveratrol and 1,25-dihydroxyvitamin D3 in a monocyte cell line could induce a synergistic effect toward the augmentation of an innate immune response [24]. Activation of sirtuins may be a mechanism explaining the function of vitamin D. Polidoro et al. showed that vitamin D can activate protective pathways in endothelial cells treated with H_2O_2, thereby inhibiting generation of superoxide, caspase, and apoptosis [25]. An et al. studied cancer chemoprotective properties of vitamin D, in which stimulation of VDR results in activation of SIRT 1, followed by deacetylation of FOXO [26]. Another, still not so detailed, interesting point is that the sirtuin class of HDACS (SIRT 1) may regulate function of VDR epigenetically [27]. Hence there might be a bilateral interaction between vitamin D and sirtuins, which needs further elucidation to provide a comprehensive insight into these two currently independent pathways in the heart. So, the current study was performed to elucidate the cardioprotective action of combined 1,25 D and a SIRT 1 activator, resveratrol, in IR injury.

In our study, resveratrol was used at 1 mg/kg/day. The first rationale for using low-dose resveratrol was

its well-known hormesis phenomenon, in which small doses of resveratrol (0.5–10 mg/kg/day) decrease the number of apoptotic cells and infarct size, leading to improvement in post-ischemic ventricular recovery. However, doses higher than 25 mg/kg/day induce cell death in cardiomyocytes [30–31]. The second reason was that we should not make any significant change in blood pressure, because cardioprotective effects of resveratrol and 1,25 D were desired, but not their hemodynamic influences.

In a similar study, 1 mg/kg/day resveratrol but not 0.1 mg/kg/day for 4 weeks showed beneficial effects on cardiac function [33]. However, duration of administration of resveratrol in our study was shorter. In a study on rabbits, neither 0.15 nor 1.5 mg/kg resveratrol before IR could decrease injury, which may be due to its single-dose nature [34]. Studies on humans have used doses of 250 and 500 mg/day. The recommended dose of resveratrol is 250 mg/day as a supplement [35, 35].

Cholecalciferol is the inactive, unhydroxylated form of vitamin D which is produced mainly in skin by photochemical conversion of 7-dehydrocholesterol or comes from *dietary* sources. Cholecalciferol is hydroxylated in the liver to become calcifediol (25-hydroxyvitamin D3). Calcifediol is again hydroxylated in the kidney to form 1,25-dihydroxyvitamin D3 (calcitriol) which is the most active hormone form of vitamin D3. In the current study, the latter form of vitamin was used.

For vitamin D, a dose was used that avoided changes in blood pressure. Some studies on rats used similar doses of 1,25 D [37]. Wong et al. showed that treatment with 0.01 μg/100 g body weight/day of 1,25 D for 6 weeks in normal rats has no effect on blood pressure, although in hypertensive rats it may strongly prevent hypertension [38]. Our study supports absence of influence on blood pressure at that dose of 1,25 D. Since the administration of 1,25 D has a direct correlation with serum level of calcium, this parameter was measured. In our study the mentioned dose of 1,25 D did not increase serum concentration of calcium significantly; so the applied dose of 1,25 D can be regarded as a so-called "non-calcemic" dose.

The dose of 1,25 D and resveratrol which was used in this study proved ineffective on blood pressure and heart rate, either individually or in combination, which shows that cardioprotective effects are independent of hemodynamic influences, and stem from direct action on cardiomyocytes.

The mechanisms responsible for cardioprotective actions of vitamin D are still to be understood. However, based on several studies, the renin-angiotensin system (RAS) is one of the main targets of this vitamin. Researchers have shown that there is an inverse relationship between the circulatory level of 1,25 $(OH)_2$ D3 and plasma renin activity, so that 1,25 D can be regarded as a "negative endocrine regulator of the RAS" [39, 40].

Effects of resveratrol on components of RAS are not well understood, but its cardioprotective action on cardiac angiotensin-induced injury, such as angiotensin-induced hypertrophy, are well-documented [41, 42]. The importance of the systemic as well as the cardiac renin-angiotensin axis on the pathogenesis of IR injury has been demonstrated in previous studies including ours [43–45]. Therefore, blockage of RAS can also be a mechanism for anti-ischemic effects of vitamin D and resveratrol, which needs more elucidation.

Another mechanism for protective actions of vitamin D and resveratrol is suppression of inflammation [46–48]. Activation of inflammatory processes in the myocardium is a source of cardiac injury under IR circumstances. Therefore, suppression of inflammation may be a common pathway for vitamin D and resveratrol in limiting IR-induced injury.

Effects on calcium homeostasis in cardiomyocytes may be another example of something in common between vitamin D and resveratrol toward augmentation of cardioprotection by each other. Resveratrol decreases arrhythmias after LAD ligation [49, 50] and also following arsenic trioxide poisoning [51]; part of this effect results from inhibition of L-type Ca^{2+} channels.

Studies on vitamin D have also shown that this vitamin affects calcium channels by non-genomic effects, in addition to affecting synthesis of proteins responsible for Ca^{2+} cycling in the myocardium through genomic influences [52, 53]. An interesting study showed that patients with congestive heart failure have had a lower vitamin D status during their earlier life compared with controls, a piece of evidence in favor of protective effects of vitamin D against cardiac dysfunction [54].

The study by Bae et al. demonstrated a significant reduction of infarct size by administration of paricalcitol as a vitamin D analogue, but they induced chronic ischemia so that infarct size was measured 4 weeks after LAD ligation, and they used the drug for 5 weeks [8].

Regarding the importance of prosurvival factors in cardioprotection against IR injury, the transcription of Trx-1, Bcl-2, and catalase as a response to either singular or combined 1,25 D and resveratrol was assessed in this study. As depicted in Figure 4, Res and 1,25 D combined increased the mRNA level of catalase considerably. In other words, decrease in infarct size was associated with

increased transcription of catalase. Therefore, it is likely that increased catalase transcription as a survival pathway inside cardiomyocytes is shared between vitamin D and resveratrol. Our study showed that combination of a non-hypotensive dose of 1,25 D and resveratrol can be a novel and effective strategy for protecting the heart against ischemia. Assessment of effects of different doses and durations of vitamin D and resveratrol on IR injury, and also elucidation of their common cellular and molecular mechanisms can lead toward using them in treatment of cardiac diseases.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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