(3R)-5,6,7-trihydroxy-3-isopropyl-3-methylisochroman-1-one reduces lipoteichoic acid-induced damage in rat cardiomyoblast cells

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Abstract

Objective: Infective endocarditis is usually caused by *Streptococcus sanguinis* and characterized by inflammatory responses in the endocardium. This study aimed to investigate if the new compound (3R)-5,6,7-trihydroxy-3-isopropyl-3-methylisochroman-1-one (TIM) isolated from *Alpinia katsumadai* Hayata could provide protection against lipoteichoic acid (LTA)-induced cell damage in embryonic rat heart cells (H9c2).
Methods: LTA-induced cell damage was established in H9c2, and the protective effects of TIM against the cell damage were examined at different concentrations (0.1–2.5 μM). The inflammatory response and oxidative stress in H9c2 cells were also measured.
Results: Treatment with TIM (0.1–2.5 μM) significantly decreased LTA-induced toxicity in H9c2 cells, which was indicated by increase in cell viability, elevation in the mitochondrial membrane potential, decrease in the release of cytochrome-c and DNA damage, inhibition of caspase-3/9 activities, and change in apoptosis-related protein expression in LTA-treated H9c2 cells. TIM treatment also significantly attenuated the redox imbalance in H9c2 cells by decreasing malondialdehyde and intracellular reactive oxygen species levels as well as by enhancing superoxide dismutase activities and glutathione levels by increasing nuclear factor (erythroid-derived 2)-like 2 protein expression. Moreover, TIM treatment decreased interleukin 1 β, interleukin 12, and tumor necrosis factor α levels by inhibiting nuclear factor kappa B protein expression.
Conclusion: Our data indicated that TIM protected H9c2 cells against LTA-induced toxicity, at least partially through inhibiting the inflammatory response and oxidative stress, providing scientific rational to develop TIM to treat infective endocarditis. *(Anatol J Cardiol 2018; 19: 198-204)*
Keywords: infective endocarditis, lipoteichoic acid, (3R)-5,6,7-trihydroxy-3-isopropyl-3-methylisochroman-1-one, inflammatory response, oxidative stress

Introduction

Infective endocarditis (IE) is the infection of the endocardial surface of the heart characterized by bacteria entering the bloodstream and settling in the heart lining, heart valve, or blood vessel, resulting in cardiac complications and embolic events (1, 2). IE is usually caused by *Streptococcus sanguinis*. However, little is known about the exact molecular mechanisms causing IE (3, 4). Current therapies include antibiotic coverage, vasopressors, and early surgery; unfortunately, there are few effective treatments available for patients with IE (2, 5, 6). Therefore, it is important to search for novel therapeutic strategies to treat IE.

Lipoteichoic acid (LTA) is a major component of Gram-positive bacteria cell membrane; it is well-known for the induction of inflammatory responses (7). LTA activates cardiomyocytes, resulting in an increased secretion of proinflammatory cytokines, including interleukin (IL)-1β, IL-12, tumor necrosis factor alpha (TNFα), and nitric oxide, by the phosphorylation of nuclear factor-κB (NF-κB) (8). The inflammatory factor over-production causes cell damage, resulting in intracellular toxic events by increasing the permeability of mitochondrial membrane, releasing cytochrome-c, activating caspase-related apoptotic proteins, and subsequently causing DNA damage and cell death (9).

Proinflammatory cytokines dramatically increase in IE. Some of these cytokines can stimulate oxidant production in the myocardium, with subsequent peroxidative damage to macromolecules with biological activities. Oxidative stress has been confirmed to play an important role in the progression of IE (10).

Supplementation of exogenous antioxidants could alleviate the oxidative damage, and different antioxidants have been used to effectively treat some disorders caused by oxidative stress (11-13). (3R)-5,6,7-trihydroxy-3-isopropyl-3-methylisochroman-1-one (TIM) is a novel compound isolated from the plant *Alpinia katsumadai* Hayata with neuroprotective effects through inhibiting oxidative stress (14). Moreover, in our preliminary work, TIM

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was found to regulate nitric oxide synthase expression and inhibit nitric oxide production. This study aimed to elucidate the protective effect of TIM against LTA-induced inflammatory response and oxidative stress in cardiomyoblasts.

Methods

Materials

H9c2 cardiomyoblast cell line was purchased from Shanghai Cell Bank (Shanghai, China). LTA was purchased from National Institutes for Food and Drug Control (Beijing, China). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's medium (DMEM) were purchased from Gibco BRL (Gaithersburg, USA). Caspase-3/9 activity assay kits and 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium-bromid (MTT) were purchased from Sigma-Aldrich (St. Louis, USA). MDA, GSH, SOD, IL-1β, IL-12, and TNFα assay kits were purchased from Jiancheng Biological Engineering (Nanjing, China). Cytochrome-c immunoassay kit was purchased from R&D systems (Minneapolis, USA). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and 2',7'-dichlorofluorescin diacetate (DCFH-DA) were purchased from Molecular Probes (CA, USA). Gamma H2AX (γH2AX) antibody was purchased from BioLegend (San Diego, USA). Real-time PCR reagents were purchased from Thermo Fisher (Waltham, MA, USA). TIM was isolated and identified by Prof. Lin from Shantou University Medical College (Shantou, China) (14). All solvents and chemicals used in this study were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). LTA and TIM were dissolved in DMSO in the *in vitro* experiments.

Cell culture and treatment

H9c2 cells were cultured in DMEM medium supplemented with 1% streptomycin–penicillin and 10% FBS in a 37°C, 95% air/5% CO_2 cell culture incubator. In the treatment experiment, H9c2 cells were incubated with TIM at 0.1, 0.5, and 2.5 μM for 4 h, followed by treatment with 15 μg/mL LTA for 24 h; DMSO was used as the negative control.

Cell viability measurement

MTT assay was used to determine cell viability. After treatment, H9c2 cells were seeded in 96-well plates at a density of 3.5×10^4/100 μL. MTT solution (10 μL) was added to each well, mixed by shaking briefly on an orbital shaker, and incubated for 4 h at 37°C. DMSO (200 μL) was added to each well to dissolve the formazan by pipetting up and down several times. The absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) plate reader, at a wavelength of 570 nm.

Measurement of mitochondrial membrane potential (MMP)

After treatment, H9c2 cells were incubated with 2 μM JC-1 for 15 min at 37°C in the dark. The fluorescent dye JC-1 labels mitochondria with a low membrane potential green and those with a high membrane potential red. Fluorescence was assessed at an excitation wavelength of 490 nm and at an emission wavelength of 590/530 nm on a fluorescence microplate reader (TECAN Polarion, UK). The change in MMP was expressed as a percentage of the negative control.

Cytochrome-c measurement

Cytochrome-c levels were measured by the assay kit, according to the manufacturer's instructions. After treatment, H9c2 cells were washed, fractionated, and incubated with the reagents. The optical density was measured on an ELISA plate reader at a wavelength of 490 nm.

DNA damage measurement

DNA damage was measured using γH2AX antibody by flow cytometry. After treatment, H9c2 cells were washed and permeabilized. After incubation with γH2AX antibody for 15 min, cells were washed and re-suspended in FACS buffer. The fluorescence was detected by flow cytometry.

Measurement of caspase activity

The caspase activities were measured by the assay kit. After treatment, H9c2 cells were harvested and 1×10^6 cells were analyzed for caspase-3 (Ac-DEVD-Amc, 390/475 nm) and caspase-9 (Ac-LEDH-Afc, 400/505 nm) activities using the fluorescent assay kit, respectively, according to the manufacturer's instruction. Caspase activity was expressed as a percentage of the negative control.

Measurement of MDA and GSH levels and SOD activity

MDA and GSH levels and SOD activity were measured by the assay kits, according to the manufacturer's instructions. Briefly, after treatment, H9c2 cells were washed twice and then homogenized. After centrifugation at 10,000 rpm at 4°C for 10 min, the supernatant was used to measure MDA and GSH levels and SOD activity, which were expressed as a percentage of the negative control.

Measurement of reactive oxygen species (ROS)

Intracellular ROS was measured by the fluoroprobe DCFH-DA. After treatment, H9c2 cells were washed twice with PBS and incubated with DCFH-DA for 30 min. After washing twice with PBS, the fluorescence intensity was measured by a fluorescence microplate reader at 488/525 nm (TECAN Polarion, UK). The ROS level was expressed as a percentage of the negative control.

Cytokine assays

After treatment, the supernatant of culture medium was collected to determine IL-1β, IL-12, and TNFα levels using ELISA kits, according to the manufacturer's instructions.

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Table 1. TIM did not cause significant toxicity in H9c2 cells (mean±SD, n=3)

		Viability
DMSO		99.67±1.53
TIM (μM)	0.1	98.17±1.15 (P=0.42)[#]
	0.5	97.50±1.29 (P=0.18)[#]
	2.5	97.25±0.96 (P=0.06)[#]

[#]vs. DMSO

Real-time RT-PCR

After treatment, H9c2 cells were collected, and the total RNA was extracted with 1000 μl TRIzol reagent. RNA (0.5 μg) was added to SuperScript master mix, and reverse transcription was performed to generate cDNA. Quantitative PCR was run on MX3000p (Stratagene) using comparative C_t value method to quantify the expression of target genes in different samples. The gene expression was normalized by the housekeeping gene β-actin. The gene-specific primer sequences are the following. For Il-1β, forward: AGGCTTCCTTGTGCAAGTGT; reverse: TGAGTGACACTGCCTTCCTG, NCBI reference: NM_031512.2. For Il-12, forward: ACCCTCACCTGTGACAGTCC; reverse: TTCTTGTGGAGCAGCAGATG, NCBI reference: NM_022611.1. For Tnfα, forward: ACTCCCAGAAAAGCAAGCAA; reverse: CGAGCAGGAATGAGAAGAGG, NCBI reference: NM_012675.3. For β-actin, forward: AGCCATGTACGTAGCCATCC; reverse: CTCTCAGCTGTGGTGGTGAA, NCBI reference: NM_031144.3.

Western blot analysis

After treatment, H9c2 cells were collected and lysed with RIPA lysis buffer. The protein concentration was determined by BCA protein assay kit (Beyotime). Samples with equal quantity (40 μg) of total protein were mixed with 4× loading buffer and subjected to electrophoresis on a 12% (v/v) SDS-polyacrylamide gel. Proteins were then transferred onto polyvinylidene fluoride membranes. After blocking with 5% dried skimmed milk, the membranes were washed thrice and incubated with primary antibodies at 4°C overnight (anti-Bcl-2 rabbit pAb ab59348, 1:1000; anti-Bax rabbit pAb ab53154, 1:1000; anti-Nrf2 rabbit pAb ab137550, 1:1500; anti-NF-κB rabbit pAb ab16502, 1:1000; anti-NADPH oxidase 4 rabbit mAb ab216654, 1:500; anti-β-actin rabbit pAb ab8227, 1:2000). After washing, the membranes were further incubated with corresponding horseradish peroxidase-conjugated secondary antibodies. The membranes were then exposed to PierceTM ECL substrates (Thermo Scientific, MA, USA) followed by X-ray film development.

Statistical analysis

Data were analyzed with SAS 9.1 software (SAS Institute, USA), and Kolmogorov–Smirnov test was used for normality test. Values were expressed as mean±SD. Dunnett's t-test was performed for comparing between the experimental and control groups. Measurement data between the two groups were compared using the t-test; measurement data among multiple groups were compared using one-way ANOVA. P<0.05 was considered to be statistically significant.

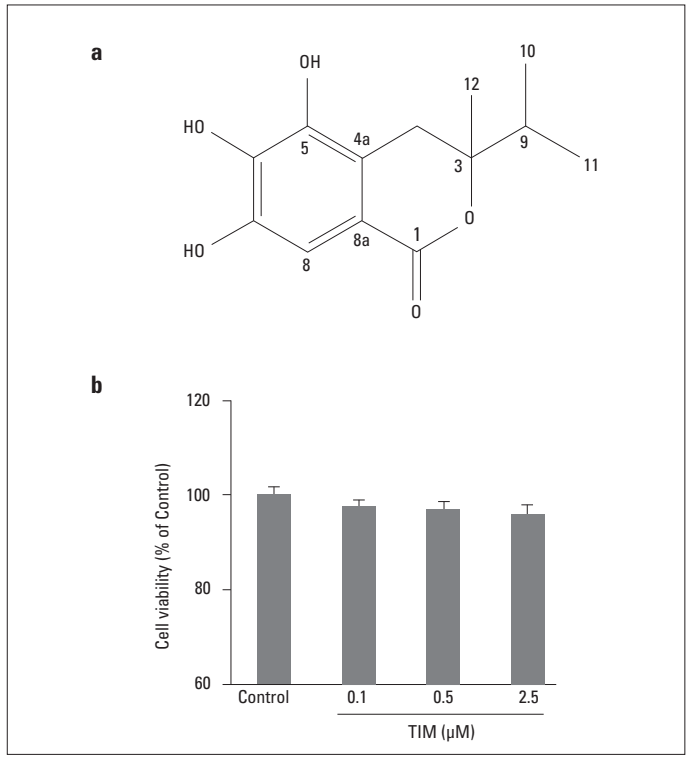

Figure 1. H9c2 cells were treated with TIM (a) at 0.1, 0.5, and 2.5 μM for 48 h, and then the cell viability was tested by MTT assay. TIM did not cause significant toxicity in H9c2 cells (b). Samples were measured in triplicate, and experiments were repeated thrice

Results

Effects of TIM on H9c2 cell viability

H9c2 cells were treated with TIM at different concentrations for 48 h. Compared with the negative control, TIM treatment did not reduce cell viability, as shown by Figure 1 and table 1.

TIM treatment protected H9c2 cells against LTA-induced cell damage

Significant toxicity on H9c2 cells was caused by LTA; however, treatment with TIM increased cell viability (Fig. 2a), together with the increase in MMP (Fig. 2b), decrease in the release of cytochrome-c (Fig. 2c), and reduction in DNA damage (Fig. 2d) and caspase activities (Fig. 2e) as well as changes in apoptosis-related protein expression (Fig. 2f) (Table 2).

TIM treatment inhibited the oxidative stress

H9c2 cells were treated with LTA for 24 h with or without TIM. The oxidative stress was measured by MDA, ROS, and GSH levels

Table 2. TIM protected H9c2 cells against the LTA-induced cell damage (mean±SD, n=3)

									Protein expression	
			Viability	MMP	Cytochrome-c	DNA damage	Caspase-3	Caspase-9	Bax	Bcl-2
DMSO			100.33±1.53	99.67±2.52	99.36±1.53	2.76±0.42	99.65±1.53	100±1.2	0.99±0.02	1.03±0.06
			58.56±4.73	58.35±6.11	155.25±9.51	16.2±2.49	224.35±13.05	197.61±13.58	1.89±0.08	0.4±0.1
			(*P*<0.001)#	(*P*<0.001)#	(*P*<0.001)#	(*P*<0.001)#	(*P*<0.001)#	(*P*<0.001)#	(*P*<0.001)#	(*P*<0.001)#
		0.1	67.15±5.51	66±5.57	139.61±9.02	14.67±2.16	200±11	161.25±10.59	1.39±0.078	1.32±0.08
			(*P*=0.36)*	(*P*=0.18)*	(*P*=0.11)*	(*P*=0.47)*	(*P*=0.18)*	(*P*=0.15)*	(*P*=0.002)*	(*P*=0.002)*
LTA (15 µg/mL)	TIM (µM)	0.5	80.35±3.79	76±4.58	126.15±8.51	10.63±1.54	170.31±7.02	142±7	1.19±0.075	2.05±0.13
			(*P*=0.03)*	(*P*=0.016)*	(*P*=0.017)*	(*P*=0.03)*	(*P*=0.031)*	(*P*=0.06)*	(*P*<0.001)*	(*P*=0.001)*
		2.5	84.69±4.51	84.32±5.03	121±7.55	7.62±1.7	146.56±5.86	25.15±6.03	0.85±0.05	2.08±0.13
			(*P*=0.001)*	(*P*=0.005)*	(*P*=0.008)*	(*P*=0.008)*	(*P*=0.014)*	(*P*=0.038)*	(*P*<0.001)*	(*P*<0.001)*

#vs. negative control; *vs. LTA alone

Table 3. TIM inhibited LTA-induced oxidative stress in H9c2 cells (mean±SD, n=3)

							Protein expression		
			MDA	ROS	SOD	GSH	Total Nrf2	Nuclear Nrf2	NADPH oxidase 4
DMSO			100.25±2.53	99.58±1.55	99.36±1.15	100.16±1.53	1.02±0.03	0.98±0.03	1.02±0.1
			175±11.79	151.33±8.5	69.35±4.51	66±5.52	0.88±0.08	0.93±0.031	2.03±0.13
			(*P*<0.001)#	(*P*<0.001)#	(*P*<0.001)#	(*P*<0.001)#	(*P*=0.047)#	(*P*=0.08)#	(*P*<0.001)#
		0.1	155±13.53	130.62±7.51	78.15±3.51	77±5.05	1.42±0.076	1.42±0.076	1.62±0.076
			(*P*=0.13)*	(*P*=0.03)*	(*P*=0.058)*	(*P*=0.06)*	(*P*<0.001)*	(*P*<0.001)*	(*P*=0.008)*
LTA (15 µg/mL)	TIM (µM)	0.5	142±11.79	123.52±6.11	84±5.57	82.67±4.16	1.44±0.05	1.44±0.05	1.53±0.08
			(*P*=0.027)*	(*P*=0.011)*	(*P*=0.026)*	(*P*=0.014)*	(*P*<0.001)*	(*P*<0.001)*	(*P*=0.004)*
		2.5	127.62±8.62	117.26±6.66	87.36±4.51	88±4.58	1.59±0.04	1.59±0.04	1.33±0.12
			(*P*=0.005)*	(*P*=0.006)*	(*P*=0.009)*	(*P*=0.006)*	(*P*<0.001)*	(*P*<0.001)*	(*P*=0.002)*

#vs. negative control; *vs. LTA alone

Table 4. TIM inhibited LTA-induced inflammatory response in H9c2 cells (mean±SD, n=3)

						Gene expression			Protein expression
			IL-1β	IL-12	TNFα	Il-1β	Il-12	TNFα	NF-κB
DMSO			100.32±2.52	99.65±2.55	99.59±1.95	1.03±0.1	1.05±0.13	0.99±0.11	1.01±0.11
			207±9	139.58±5.69	145±9.17	8.56±0.83	8.43±0.97	8.7±0.8	1.45±0.12
			(*P*<0.001)#	(*P*<0.001)#	(*P*=0.001)#	(*P*<0.001)#	(*P*<0.001)#	(*P*<0.001)#	(*P*=0.009)#
		0.1	195.32±9.5	131.35±5.86	135.25±6.51	8.83±0.61	6.75±0.8	7.8±0.6	0.84±0.08
			(*P*=0.19)*	(*P*=0.15)*	(*P*=0.21)*	(*P*=0.68)*	(*P*=0.08)*	(*P*=0.21)*	(*P*=0.002)*
LTA (15 µg/mL)	TIM (µM)	0.5	178±8.54	123±4.58	126±5	6.8±0.53	5.91±0.85	6.6±0.75	0.63±0.096
			(*P*=0.016)*	(*P*=0.017)*	(*P*=0.034)*	(*P*=0.036)*	(*P*=0.028)*	(*P*=0.03)*	(*P*<0.001)*
		2.5	167.65±8.51	116±5.57	118±4	5.63±0.55	5.15±0.51	5.63±0.67	0.48±0.075
			(*P*=0.005)*	(*P*=0.007)*	(*P*=0.009)*	(*P*=0.007)*	(*P*=0.007)*	(*P*=0.007)*	(*P*<0.001)*

#vs. negative control; *vs. LTA alone

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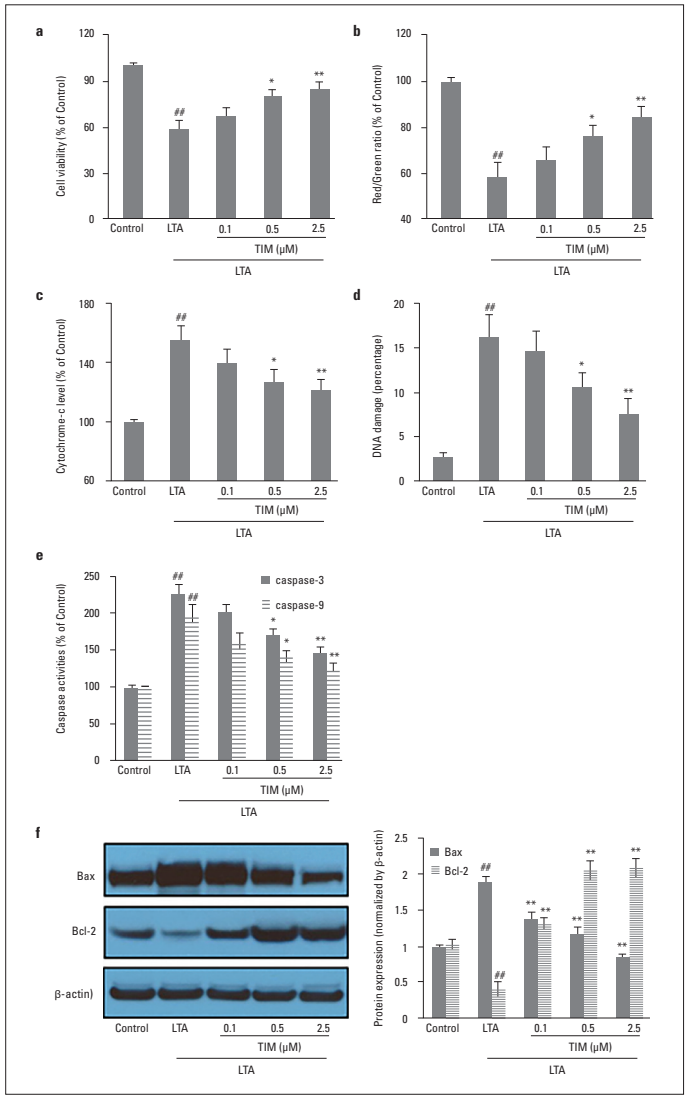

Figure 2. TIM protected H9c2 cells against LTA-induced cell damage. H9c2 cells were treated with TIM at 0.1, 0.5, and 2.5 μM for 4 h, followed by treatment with LTA for 24 h. Cells were collected to measure the cytotoxicity. Cytotoxicity was determined by cell viability (a); mitochondrial membrane potential (b); cytochrome-c releasing (c); DNA damage (d); caspase activities (e) and apoptosis-related protein expression (f). ##*P*<0.01 vs. negative control; **P*<0.05, ***P*<0.01 vs. LTA alone. Samples were measured in triplicate, and experiments were repeated thrice

as well as by SOD activity and protein expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4. Compared with negative control, oxidative stress was significantly induced after LTA treatment. However, treatment with TIM significantly reduced MDA and ROS levels and increased SOD activity and GSH levels, together with decreasing the protein expression of NADPH oxidase 4 and increasing the protein expression of both total and nuclear Nrf2, indicating that TIM effectively inhibited oxidative stress (Fig. 3 and Table 3).

TIM decreased the inflammatory response in H9c2 cells

H9c2 cells were treated with LTA for 24 h with or without TIM. Compared with LTA alone, treatment with TIM significantly de-

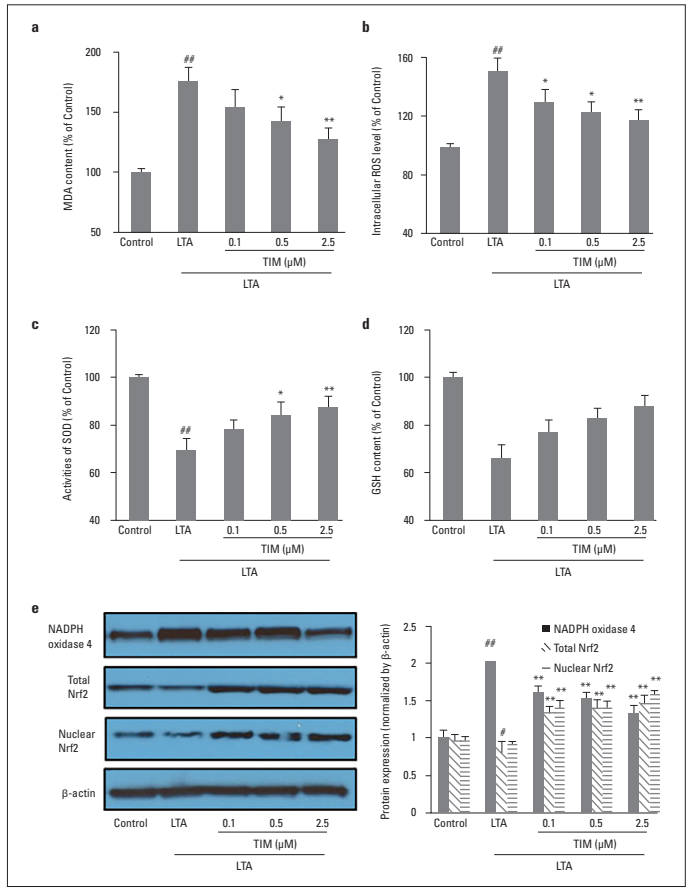

Figure 3. TIM inhibited LTA-induced oxidative stress in H9c2 cells. H9c2 cells were treated with TIM at 0.1, 0.5, and 2.5 μM for 4 h, followed by treatment with LTA for 24 h. Cells were collected to measure the oxidative stress markers: MDA content (a); intracellular ROS level (b); SOD activity (c); GSH level (d); and oxidative stress-related protein expression (e). ##*P*<0.01 vs. negative control; **P*<0.05, ***P*<0.01 vs. LTA alone. Samples were measured in triplicate, and experiments were repeated thrice

creased IL-1β, IL-12, and TNFα levels in the supernatant as well as their mRNA expression, together with the decrease of NF-κB protein expression (Fig. 4 and Table 4).

Discussion

In this study, we demonstrated that a novel antioxidant from herbal showed no toxicity in rat cardiomyoblast cells and protected H9c2 cells against LTA-induced cell damage by inhibiting inflammatory response and oxidative stress. Cardiomyocytes produce many proinflammatory cytokines in inflammatory response, and chronic inflammatory response causes oxidative stress, resulting in severe organ damage and IE (15-17). Therefore, reducing chronic inflammatory response and inhibiting oxidative stress are effective strategies to prevent pathological progression.

Mitochondria play an important role in cell death regulation (18). Decrease in MMP induces the release of cytochrome-c from the mitochondria to nucleus and activates caspase-relat-

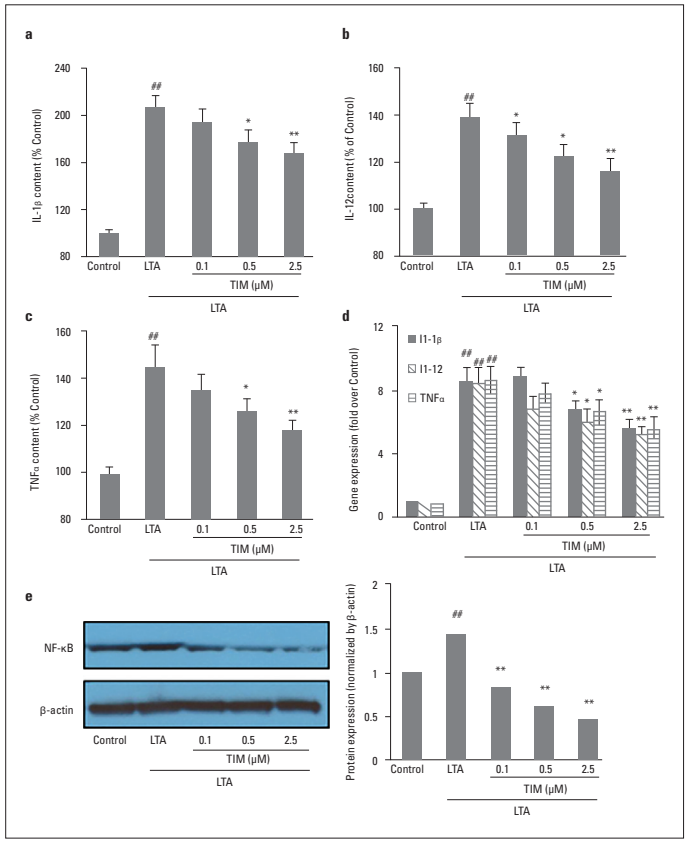

Figure 4. TIM inhibited LTA-induced inflammatory response in H9c2 cells. H9c2 cells were treated with TIM at 0.1, 0.5, and 2.5 μM for 4 h, followed by treatment with LTA for 24 h. Cells were collected to measure the inflammatory response: IL-1β content (a); IL-12 content (b); TNFα content (c); gene expression (d); and NF-κB protein expression (e). ##P<0.01 vs. negative control; *P<0.05, **P<0.01 vs. LTA alone. Samples were measured in triplicate, and experiments were repeated thrice

ed apoptotic proteins, resulting in chromatin condensation and DNA damage. Measuring double stranded breaks has been of interest in the research because of its prediction of toxicity in cells (19, 20). As an apoptotic executor, caspase-3 together with caspase-9 precursor activates endonucleases and cleaves nuclear DNA, ultimately leading to cell death (21). In this study, treating H9c2 cells with LTA caused cell damage, characterized by decrease in MMP, release of more cytochrome-c, DNA damage, and increase in caspase-3/9 activities. Anti-apoptotic protein Bcl-2 expression decreased, and pro-apoptotic protein Bax expression increased. However, the above effects induced by LTA were largely reversed by treatment with TIM.

Oxidative stress affects various biological macromolecules and impairs cellular function (22-24). This type of damage has been considered as a major cause of cellular injuries in some disorders (25-27). Therefore, supplementation of external antioxidants to eliminate ROS is required to reverse the imbalance between the intracellular oxidative and anti-oxidative systems, as a potential therapy. ROS generation has been widely implicated in the process of cell death (28). Nrf2 is a transcription factor involved in cellular defense against oxidative stress, which remains inoperative in the cytoplasm by binding to Keap1 (29). Upon activation, Nrf2 is released from Keap1 and moves into the cell nucleus, binds with antioxidant response element, and induces cytoprotective target protein expression, such as phase II detoxifying enzymes, antioxidant proteins, and molecular proteasome/chaperones (30, 31). In this study, TIM-induced activation of Nrf2 was observed, which would subsequently trigger expression of antioxidant genes to restore oxidative homeostasis, as evidenced by the markedly decreased protein expression of NADPH oxidase 4, reduced levels of MDA and ROS, and enhanced SOD activity and GSH level. The protective effects of TIM might be attributed to the hydroxyl groups with powerful free radical-scavenging ability.

Inflammatory cytokines such as TNFα, IL-1β, and IL-12 are important mediators in the progress of inflammatory diseases (32, 33). The production of these cytokines participates in the immune response to many inflammatory stimuli. NF-κB has been considered as a prototypical proinflammatory factor, largely based on the activation of NF-κB by inflammatory cytokines such as TNFα and IL-1β, and the role of NF-κB in the gene expression of other proinflammatory mediators (34). Our results showed that treating H9c2 cells with LTA induced gene expression of IL-1β, IL-12, and TNFα, and resultantly increased their levels in the supernatant. However, treatment with TIM significantly reversed the LTA effects. Additionally TIM inhibited protein expression of NF-κB, implying that TIM regulated the expression of other inflammatory molecules through NF-κB.

Study limitations

One limitation of this study is the use of cell line in the *in vitro* study, which is far from the real situation and can only be used as the lead compound screening. Hence, a corresponding *in vivo* study is required. The exact mechanisms of TIM against LTA-induced cell damage should be further explored.

Conclusion

Herbal plants have been confirmed to be an important source of medicinal products. Our study demonstrated that TIM isolated from *A. katsumadai* Hayata inhibited LTA-induced inflammatory responses and oxidative stress in cardiomyoblasts, providing the scientific rationale to develop TIM as a therapeutic agent in inflammatory diseases, including IE.

Conflict of interest: None declared.

Peer-review: Externally peer-reviewed.

Authorship contributions: Concept – Z.L.; Design – Z.L., Z.W.; Supervision – Z.L.; Fundings – Z.L.; Materials – Z.L.; Data collection &/or processing – L.X., T.B.; Analysis &/or interpretation – L.X., G.Q.; Literature search – Z.L., Z.W.; Writing – Z.L., L.X.; Critical review – Z.L.

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