ORIGINAL ARTICLE

Gallic Acid and Cyclosporine Mixture and their Effects on Cardiac Dysfunction Induced by Ischemia/Reperfusion and eNOS/iNOS Expression

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Abstract

Background: Although many researches have been conducted on either a certain antioxidant or mPTP individually, little attention has been drawn to the effects of co-administration of an antioxidant and mPTP inhibitor together on cardiac dysfunction after I/R injury.

Objectives: This study aims at determining the effects of gallic acid (as Antioxidant) combined with cyclosporine A (CsA) (as mPTP inhibitor) on I/R induced cardiac and endothelial (role of NO) dysfunction.

Methods: Male Wistar rats were pretreated with gallic acid (7.5, 15, or 30 mg.kg-1 body weight, daily) for a period of 10 days. Then, the heart was isolated and exposed to 30-minute ischemia and perfused by CsA (0.2 µM) 20 min during reperfusion period.

Results: The data have shown that infarct size was decreased significantly by CsA and gallic acid alone ($p < 0.05$, one way ANOVA followed by LSD test), however the combination of both drugs had more significant improving effects ($p < 0.001$). The combination of these two drugs improved more significantly maximum rate of rise and fall of ventricular pressure (±dp.dt-1 max), rate pressure product (RPP), left ventricular developed pressure (LVDP), heart rate and coronary flow rather than applying each one alone ($p < 0.05$, repeated measurement ANOVA followed by LSD test).

Conclusions: In conclusion, benefiting from an antioxidant concomitant with an mPTP inhibitor could have more improving effects on the cardiac dysfunction induced by I/R injury. (Int J Cardiovasc Sci. 2017;30(3):207-218)

Keywords: Antioxidants; Gallic Acid; Cyclosporine; Ventricular Function; Endothelium; Reperfusion; Rats; Nitric Oxide Synthase.

Introduction

The disrupted supply of oxygen accompanied by myocardial ischemia leads to mitochondrial decline in electron transport chain (ETC) flux whose result is the subsequent mitochondrial swelling and degeneration. Then, ATP is depleted, so necrotic and mitochondrial death pathway occurs with the opening of mitochondrial permeability transition pore (mPTP).[1-3]

The concept of myocardial cell injury occurring after ischemia/reperfusion (I/R) involves two major hypotheses: increases in intracellular calcium and/or the accumulation of reactive oxygen species (ROS). The latter causing the sarcolemmal peroxidation of the cellular phospholipid layer, leads to the loss of cellular integrity and facilitating calcium entry. During reperfusion, calcium enters the mitochondria in response to a variety of stimuli and can modulate the opening of the mPTP shown with its accessory components.[2,4]

NO is an important vascular tone modulator. Functional damage in the endothelium due to peroxynitrite formation is the aftermath of combining ROS with NO. This toxic compound can result in the initiation of apoptotic cell death.[5] Prior studies

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revealed that red wine extract and antioxidant treatment significantly improved post ischemic ventricular and contractil function.[6,7] To reduce lethal reperfusion injury, new cardioprotective strategies, including preconditioning with antioxidants and inhibitors of the Mptp,[8] have been applied. One of the fundamental mechanism involving the opening of K^+_{ATP} channels in the mitochondria results in the blockade of ischemia-induced collapse of the mPTP, and thereby leads to the preservation of mitochondrial function and cellular ATP.[8,9]

Previous studies have demonstrated that gallic acid, a metabolite of propyl gallate, improves antioxidant status and protects the heart and lysosome membrane against isoproterenol-induced oxidative stress in rats.[10] Moreover, the perfusion of polyphenol (-)-epigallocatechin gallate (EGCG) during ischemia significantly reduced infarct size (50%) after reperfusion. It should be noted that the mitochondrial K^+_{ATP} channels played a crucial role in cardioprotection induced by EGCG.[11] Gallic acid treatment, showed in Ananya (2012), improved ulcer healing by increasing eNOS/iNOS ratio.[12] In another study, Gallic acid induced eNOS expression and modulated the endothelium-dependent vasodilation and vasoconstriction.[13] eNOS with anti-inflammatory role could protect the cell against I/R injury by increasing the NO production.[13]

However, literature survey has shown that there are no scientific reports available on the antioxidant effects of gallic acid on hemodynamic parameters or NOS expression after I/R injury.

In addition, it has been unraveled that the inhibition of mPTP by cyclosporine A preserved mitochondrial morphology after myocardial ischemia/reperfusion, limited myocyte necrosis and apoptosis, and reduced infarct size (60%).[14]

In accordance with the aforementioned facts, oxidative-stress-induced opening mPTP casts a pivotal role in lethal perfusion injury which is a cornerstone in cardioprotection. Therfore, the main object of this study was to investigate the effect of combination of gallic acid as a powerful antioxidant and cyclosporine A as a mPTP inhibitor on cardiac hemodynamic dysfunction, infarct size and the NOS expression after ischemia/reperfusion in rats' isolated heart.

Material and Methods

Ethical approval

The protocols and procedures of the present study were approved by the animal care and use committee of the Ahvaz Jundishapur University of Medical Sciences (AJUMS.REC.1392.222).

Animals

The experiments were conducted on ninety male Wistar rats weighing (250-300g) obtained from animal house of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. The animals were randomly divided into the following 9 groups (Table 1). In each group 10 rat were chosen using Cochran formula. Different doses of gallic acid were dissolved in saline and administered once a day for 10 days orally via a gavage needle.[10] All groups were maintained under the same conditions (temperature controlled room 22±2°C, with a 12 h dark- light cycle supplied with food and water ad libitum).

We chose the sample size using Cochran formula. With using it the number of rat in each group was____. But we predicted some missing because of ischemia. So we selected 10 rat in each group.

Cochran formula was:

$$n = \frac{Z^2_{\frac{\alpha}{2}} \times S^2}{d^2}$$

Table 1 – Animals groups that have used in this study (n= 10)			
Groups[1]		**Gallic acid (mg/kg)**	**Cyclosporine (µM)**
Sham[2]			
C1	C_a	Saline	0
	C_b	Saline	0.2
G1	$G1_a$	7.5	0
	$G1_b$	7.5	0.2
G2	$G2_a$	15	0
	$G2_b$	15	0.2
G3	$G3_a$	30	0
	$G3_b$	30	0.2

C: control groups; G: groups that received gallic acid. This group wasn't exposed to ischemia / reperfusion.

Drugs

Gallic acid , cyclosporine A (CsA), heparin, 2,3,5-triphenyl tetrazolium chloride (TTC), were purchased from Sigma (St. Louis, MO); sodium chloride, potassium chloride, magnesium sulphate, sodium hydrogen carbonate, potassium hydrogen orthophosphate, D-glucose and calcium chloride were obtained from Merck Laboratories, ketamine and xylazine from Alfasan Co (Woderen- Holland).

Isolation and preparation of the heart

The animals were anesthetized by Ketamine HCL (50 mg.kg^{-1}), xylazine (2-5 mg.kg^{-1}) and to prevent blood clotting heparin (1000 U.kg^{-1}, IP) was injected. The trachea was cannulated and the animals were ventilated with room air using a rodent ventilator (UGO BASILE, model: 7025).[15] The chest was opened and the ribs were removed, and then a steel cannula was placed into the aorta and secured with a suture. The heart was immediately perfused with Krebs-Henseleit solution. Then, the heart was quickly excised and transferred to a Langendorff apparatus while it was continuously perfused with the solution at a constant (60-70 mmHg) pressure and temperature (37°C). Before starting the experiments, the buffer was bubbled with 95% O2-5% CO2 to attain a pH of 7.4.

A water-filled latex balloon was connected to a pressure transducer by a stainless steel needle was introduced through the left atrium into the left ventricle to measure left ventricular pressure (LVP). The heart was placed in a jacketed glass chamber at 37°C temperature and provided with a 25-to 30-min period to reach equilibrium before any experiment. The balloon volume was adjusted in a way that a LV end diastolic pressure (EDP) reaches to 5-10 mmHg. A Power lab system (Power lab, AD instrument, Australia) was used to analyze the signal from the pressure transducer. Left ventricular developed pressure (LVDP), left ventricular systolic pressure (LVSP), ventricular EDP, maximum rate of rise (+dp.dt[-1] max) and maximum rate of fall (-dp.dt[-1] max) of LVP, and the rate-pressure product (RPP; product of LVDP and heart rate) and lead II of ECG were recorded. All hearts were perfused for 25-30 min before the induction of ischemia to allow the stabilization of LVP and Coronary Perfusion Pressure (CPP). Next, they were subjected to a 30-min no flow global ischemia, followed by 60 min of reperfusion.[16] The successful induction of ischemia was determined by ST elevation on the electrocardiogram. Heart rate and perfusion pressure were constantly monitored.[15] CsA was dissolved in ethanol and added to perfusion solution. After 30 min of ischemia, the hearts were reperfused with 0.2 μM of CsA for 10 min and then the reperfusion continued with ordinary Krebs-Henseleit solution buffer.[17] The above mentioned cardiac parameters were measured at the end of ischemia and at every 15 minutes during reperfusion period.

Determination of infarct size

At the end of the above mentioned experiments, the heart was sliced (2 mm thick) and incubated with 1% TTC for 20 minute at 37°C temperature. Then, the slices were incubated in 10% formalin for 60 minutes. Next, the infarct area was measured by calculating the percentage of the total area of slice on both sides and via using image analysis software (NIHimagepro.1.16).[17]

Evaluation of eNOS mRNA expression

Tissue samples were frozen for 1 month. On the experimental day, the total RNA was extracted from the frozen tissue samples using *Trizole* reagent (Roche Diagnostics, Indianapolis, IN). The concentration of the total RNA was determined by spectrophotometry (*Bio Photometer Plus*, Eppendorf, Hamburg, Germany). We read the RNA at 230 and 260nm. The samples which were in 1.9-2.1 mm were selected to continue the assessment. The others were excluded of the study.

The cDNA, according to the manufacturer's instruction, was synthesized from 1 μg of the total RNA with the *Reverse Transcription* kit (Fermentase, England).

Reverse-transcribed polymerase chain reaction (RT-PCR) was performed to determine mRNA levels of the constitutive (endothelial) (eNOS) and inducible nitric oxide synthase (iNOS) isoforms in rats' ventricular tissue.[18]

PCR amplification

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified from the same amount of RNA to evaluate a variety of different samples in comparison to the control group. The PCR solution contained 10 μl of first-strand cDNA, 5μl 10X PCR buffer, 10mM MgCl2, 25 Pico mole of both sense (5_-ACC ACA GTC CAT GCC ATC AC-3_) and antisense (5_-TCC ACC ACC CTG TTG CTG TA-3_) GAPDH primers.[19] It yielded 400 bp of GAPDH, 25picomole of both sense (5_-CGA GAT ATC TTC AGT CCC AAG C-3_) and antisense

(5_-GTG GAT TTG CTG CTC TCT AGG-3_) eNOS primer,[19] which yielded 200 bp of eNOS, and 25Pico mole of sense (5`CACCTTGGAGTTCACCCAGT3)`and antisense (5`ACCACTCGTACTTGGGATGC3) iNOS primer,[20] which yielded 220 bp of iNOS message, 5U/µl Termophylus Acquaticus (Taq) DNA polymerase (Cinagen, Iran,), and water to a final volume of 50 µl.

The cycling program consisted of incubation at 95˚C for 3 min (eNOS and GAPDH) and at 94˚C for 45s for (iNOS) to active DNA Taq polymerase and 35 two-step cycles with denaturation at 60˚C for 60 s (eNOS and GAPDH) and 60˚C for 45s (iNOS) and annealing/extension at 72˚C for 10 min for all of them. The template negative control (H2O) was routinely run in every PCR. PCR products were run on 2% agarose gel electrophoresis and photographed after ethidium bromide staining under UVlight. Expression levels of all genes were normalized against GAPDH expression (internal calibrator for equal RNA template loading and normalization). Using a computerized densitometric system (Image J),[18] bands on the gel were scanned and quantified.

Statistical analysis

The results were analyzed by using SPSS version 16 and expressed as Mean±SEM. Comparisons between groups were performed using one-way ANOVA or repeated-measurement ANOVA followed by LSD multiple comparison test. P-values of less than 0.05 were considered significant. Normality was checked using Kolmogorov Smirnov test. We accessed the hemodynamic changes between control group and others during the 2 hours of test. This time included the pre ischemia period, ischemia and reperfusion. During this time we recorded the changes repeatedly. The main aim was to compare this changes between control and treated groups. So comparison within each group was not performed.

Results

Effects of Gallic Acid +CsA on myocardial function

Myocardial contractility (shown as $\pm dp.dt^{-1}$) was decreased in almost all groups, as shown in Table 2. Although pretreatment with gallic acid (7.5–30 mg.kg^{-1}) alone had no significant effects, CsA improved the contractility significantly compared with control ($p<0.01$). Nevertheless, the combination of gallic acid (15 mg.kg^{-1}) and CsA had more significant

improving effects on the contractility ($p<0.001$). Also the combined form improved contractility compared with only CsA ($p<0.05$) and no significant effect with gallic acid alone.

Furthermore, different doses of gallic acid or CsA alone had no significant effects on LVDP, while the administration of gallic acid (7.5 and 15 mg.kg^{-1}) + CSA improved LVDP significantly ($p<0.05$, Table 2). The LVSP was increased by gallic acid (7.5 and 15 mg.kg^{-1}) pretreatment in combination with CsA ($p<0.05$); but the combined effect of 15 mg.kg^{-1} gallic acid + CsA was more significant than that of gallic acid (7.5 mg.kg^{-1}) pretreatment + CsA compared with control ($p<0.01$, Table 2). This rising was not significant in comparison with CsA or gallic alone.

The rate pressure product (RPP) was decreased in almost all groups, as shown in Table 2. Although pretreatment with gallic acid (7.5–30 mg.kg^{-1}) alone had no significant effects on RPP, the combination of gallic acid (15 +CsA mg.kg^{-1}) and CsA improved it significantly compared with control ($P<0.001$). In this case, comparison of combinations treats with CsA or gallic alone was not significant.

Coronary flow

Coronary flow decreased in all groups during the post-ischemic reperfusion compared to pre-ischemia. The administration of gallic acid (15mg.kg^{-1}) + CsA and CsA improved the coronary flow significantly toward normal pre-ischemic value compared with control (Table 2). However, other doses of gallic acid alone or in a combined form had no significant effects on the coronary flow compared with control or CsA alone. Also the gallic acid alone had not significant effect in comparison with its combined form.

Heart rate

At the beginning of the experiment, the rate of isolated heart in all groups was not different from one another (pre ischemia period). However, at the end of reperfusion, the heart rate of all groups was reduced significantly compared with control ($p<0.05$, Table 2) but this reduction was not significant in comparison with CsA or gallic acid alone. Nevertheless, the administration of CsA alone increased the heart rate slightly during the first 60 minutes of reperfusion.

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Table 2 – Hemodynamic parameters during pre-ischemia and reperfusion (60 min of post ischemia) periods of ischemia-reperfusion protocol

Group[1]		+dp/dt	-dp/dt	LVDP	LVSP	RPP	CF	HR
Ctrl	Pre	2991.2±384	1615.5±237.5	90±15.1	95.5±15.2	27989.5±5499.9	11.2±1	273.7±12.6
	Post	605.6±72.3	291.7±37.4	22.9±2.9	33.3±5.3	4319.2±394	4.1±0.5	171.2±15.6
G 7.5	Pre	2342.7±278.9	1067.4±152.4	68.4±9.1	74±9.1	18084.8±3672.1	11.8±1.1	236±10.5
	Post	1358.3±546.9	504.2±215.6	41.2±14.3	59.5±11.5	8970.4±3516.9	6.2±1.5	200.8±21.9
G 15	Pre	2801.2±312.6	1242.7±131.5	67.3±5.1	74.2±5.2	19349.7±1273.5	11.7±0.8	281.3±9.1
	Post	820.5±134	321.7±59.6	25.2±3.4	37.5±4.2	3934.6±903.7	5.4±1	152.8±18.3
G 30	Pre	2367.3±210.8	1507.9±568.7	64.4±7.9	71.9±7.8	18409.3±2323.4	10.3±1	257.7±13.3
	Post	840±141.4	306.9±56.3	39±8.3	51.8±7	6271.9±2153.9	5±0.6	151.6±24
CsA	Pre	3036.4±374.5	1470.2±236.2	82.1±10.2	88.1±9.9	23409.6±2442.3	10.4±1.2	292±14
	Post	1510±319.9**	670±142.1	36.3±7.6	53.5±7.1	8881±1623.9	7.6±0.9* (p=0.006)	210.9±19.1
G 7.5+ CsA	Pre	2477.9±192.7	1165.6±168.6	66.6±5	72.1±5.7	15614.3±1681.6	10.4±1.1	234±16.6
	Post	1016.6±225.9	402.8±128.8	63.8±7** (p=0.003)	73.8±20.1* (p=0.01)	11486.5±5450.9* (p=0.02)	5.3±1.3	193.9±19.6
G 15+ CsA	Pre	2764.9±208	1614.4±213.5	89.1±13	94±12	23173.8±3674.2	10.7±1.2	243.6±9.2
	Post	2142.1±314.9**, # (p=0.007)	841.4±109.5*,# (p=0.03)	54.1±8.3** (p=0.007)	88.8±10.3** (p=0.002)	10063±1601.6** (p=0.001)	7.7±1.9** (p=0.007)	187±17.2
G 30+ CsA	Pre	2554.4±180	1164.4±123.9	72.5±7	78.9±7.2	20498±2285.7	8.6±1	283.2±17.7
	Post	911.3±198.2	327.6±77.4	22.6±4.4	35.8±6.1	4462±1050.4	6.5±1.5	186.8±24

[1] Ctrl: control group, G7.5: gallic acid (7.5 mg/kg), G15: gallic acid (15 mg/kg), G30: gallic acid (30 mg/kg), CsA: cyclosporine A, G7.5+CsA (gallic acid (7.5 mg/kg)+ CsA), G15+CsA (gallic acid (15 mg/kg)+ CsA), G30+CsA (gallic acid (30 mg/kg)+ CsA), +dp/dt: Maximum rate of rise of left ventricular pressure, - dp/dt: Minimum rate of rise of left ventricular pressure, LVDP: left ventricular developed pressure, RPP: rate of pressure product, CF: coronary flow HR: heart rate, pre: pre ischemia, post: post ischemia.
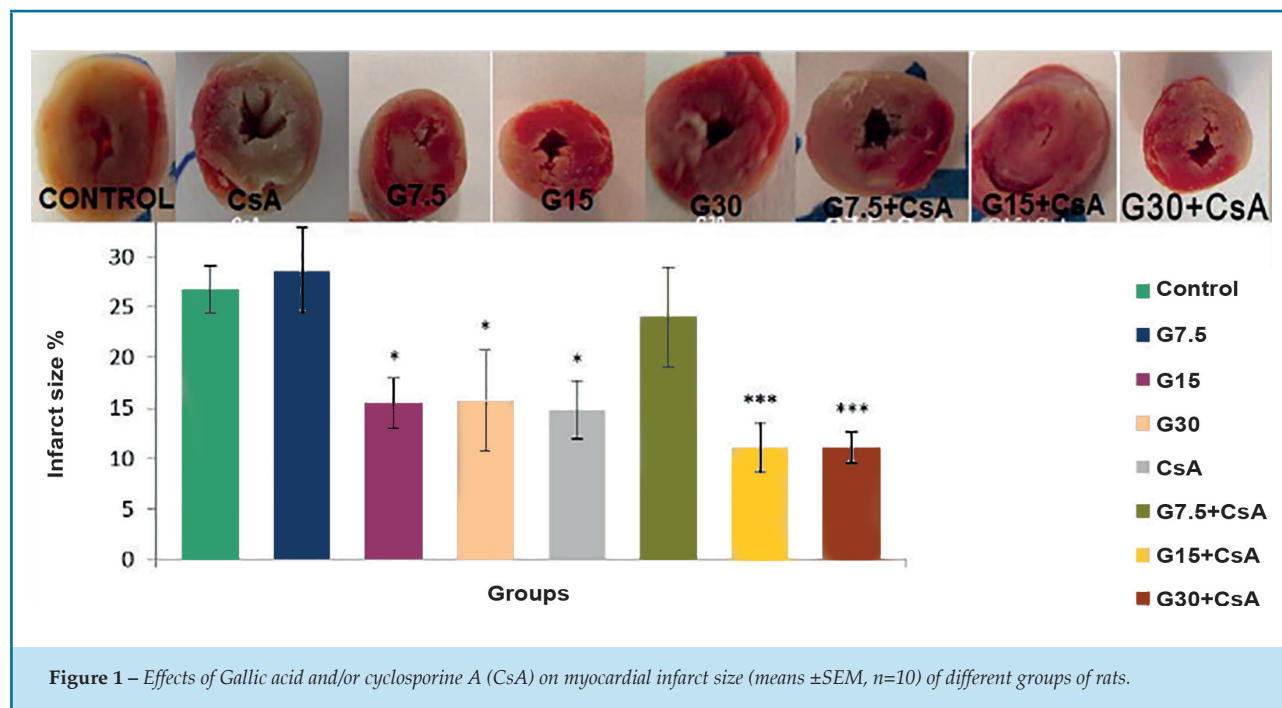* p < 0.05: compared with control, ** p < 0.01: compared with control, *** p < 0.001: compared with control. #: compared with CsA alone.

Effects of Gallic Acid +CSA on myocardial infarct size

Myocardial infarct size was expressed as the percentage ratio of the infarct area to the total area. This ratio was 26.74 ± 2.3 % for the control group subjected to 30 min of ischemia and 1h of reperfusion (Figure 1). In spite of the fact that there was a significant reduction in the infarct size for those groups that received gallic acid 15,30mg.kg [-1] or CsA alone (p<0.05, one way ANOVA followed by LSD), this reduction for those groups receiving gallic acid (15 or 30 mg.kg [-1] doses) +CsA was much more significant compared to control (p<0.001, one way ANOVA followed

by LSD). There was no significant decrease between combined form compared with CsA or gallic acid alone.

The animals received three different doses of gallic acid (7.5, 15 and 30 mg/kg) for 10 days prior to isolation of the heart and induction of ischemia (30 min) and reperfusion (1 h) without or with CSA (0.2 µM, 10 min before and during reperfusion). Control animals received normal saline as the solvent of gallic acid. Myocardial infarct size expressed as the ratio of the infarct area to the total risk area in percentage.* Indicates significant difference with control group (*p<0.05, *** p<0.001, n=10 per group one way ANOVA followed by LSD test).

Figure 1 – *Effects of Gallic acid and/or cyclosporine A (CsA) on myocardial infarct size (means ±SEM, n=10) of different groups of rats.*

Effects of Gallic Acid and Cyclosporine on eNOS and iNOS mRNA expressions

The analysis of PCR results revealed that ischemia / reperfusion decreased the levels of mRNA expression of eNOS in control group in comparison with Sham operated rats, but mRNA expression of iNOS increased in control group compared to sham. The mRNA levels of eNOS were significantly increased in gallic acid-treated (G7.5, G15 and G30 mg/kg) animals or those treated with CsA (0.2μM) alone, compared to the control group (*p*<0.001; Fig. 2a). The highest levels of mRNA expression of eNOS were observed in the administration of concomitant gallic acid and CsA compared with control. (*p*<0.001; Fig. 2a). The mRNA expression elevation was not significant in comparison with CsA or gallic alone.

iNOS mRNA expression was decreased significantly using gallic acid (G15 and G30 mg/kg) or CsA alone compared to that of control group, however the combination of both drugs induced more reduction with lower doses of gallic acid (G7.5+CsA , G15 + CsA) (*p*<0.001; Fig. 2b) and supplementary figure. The mentioned changes were not significant in comparison with CsA or gallic alone.

Data was expressed as the mean ± SEM. The animals received three different doses of gallic acid (7.5, 15 and 30 mg.kg-1) for 10 days prior to the isolation of the heart and the induction of ischemia (30 min) and reperfusion (1 h) without or with CSA (0.2 μM, 10 min before and
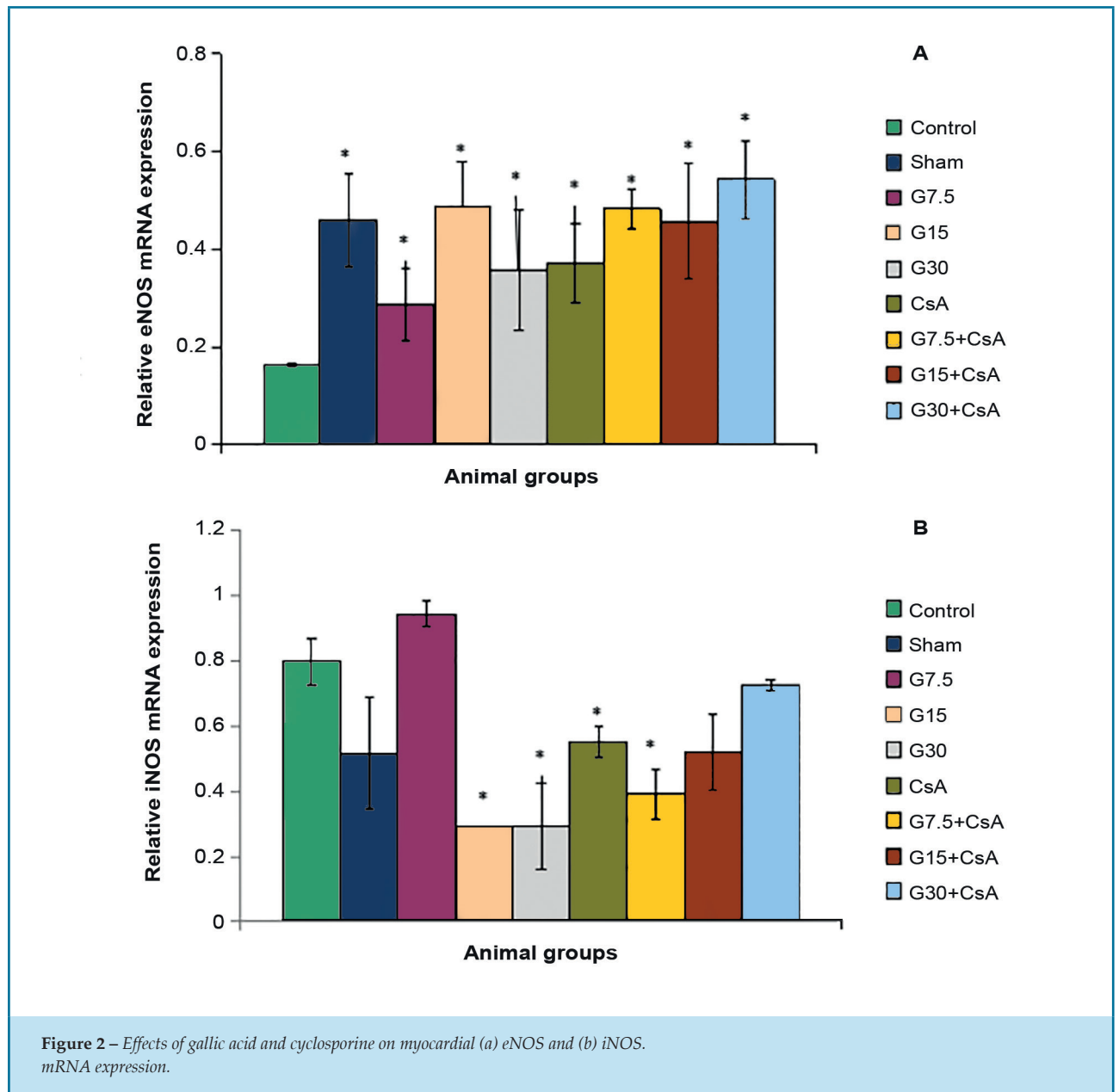
during reperfusion). Analysis of quantitative PCR results revealed that the administration of gallic acid reduced mRNA expression of iNOS but it increased that of eNOS.

For the description of experimental groups, see caption to Figure 2a, and b. Asterisks indicate a significant difference. ***p<0.001 as compared to the control group (n=6).

Discussion

As expected, the cardiac functions such as contractility, RPP, coronary flow and LVDP reduced during ischemia and reperfusion. The results of this study have shown that although the pretreatment of animals with gallic acid alone, as an antioxidant, reduced the infarct size, it had not significant effects on cardiac functions. Furthermore, the reperfusion of the heart with CsA improved cardiac functions partially, and reduced the size of infarction. Nevertheless, the combination of both drugs had more significant effects on the cardiac performance and the reduction of infarct size.

The fourth object was measuring the infarct size. Other studies showed that this parameter increased after 3 hours of reperfusion compared with 1 hour[21] with TTC staining. So we evaluated it after 1 hour of reperfusion in according with our study main objects (investigation the changes during early of reperfusion not late).

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Figure 2 – *Effects of gallic acid and cyclosporine on myocardial (a) eNOS and (b) iNOS.*
mRNA expression.

Under normal conditions, there is a balance between the formation of pro-oxidants and the amount of antioxidants. During I/R, the impairment of myocardial function is mainly attributed to the interruption of the aforementioned balance and the elevation of ROS production.[22] It has been shown that the excessive ROS could lead to peroxynitrite formation from NO radicals in response to its combination with superoxide anion and damage the cells in vascular and cardiac tissues.[23] Furthermore, lipid peroxidation of cell membrane exacerbates the cell energy depletion due to the damage of mitochondrial enzymes. In addition, nucleic acids, cell membrane integrity, sarcoplasmic reticulum (SR) and Na^+-Ca^{2+} exchanger function are damaged, so that intracellular and mitochondrial $Ca2^+$ overloads occur.[24] Following these events, the excess of Ca^{2+} induces mitochondrial enzymes dysfunction, membrane depolarization and opens the mPTP in the heart myocytes.[2] During ischemia, because of disruption of electron transport chain (ETC), ATP production is impaired and the cell is depleted of high energy phosphates.[25] Following these injuries, due to anaerobic metabolism and the accumulation of lactate and pyruvate, the intracellular acidosis is increased,

and then the uncoupling of oxidative phosphorylation occurs which, in turn, leads to mitochondrial swelling. This vicious cycle leads to necrotic cell death and cardiac dysfunction.[26] After the opening of the mPTP, cytochrome C is released into the cytosol, interacts with apoptosis protease activating factor-1(APAF-1) and leads to the activation of the caspase-9 which activates caspase 3 that is a key executor for apoptotic signaling.[27]

During the last few years, some cardio protection strategies have been applied to reduce the lethal injuries of I/R, including pre- and post-conditioning by antioxidants, by activators of the reperfusion injury salvage kinase pathway (RISK), by inhibition of protein kinase c-delta and ultimately by mPTP inhibitors such as CsA or sanglifehrin A.[28] In this study, our strategy was to protect the heart by using antioxidant and prevent from opening the mPTP. By means of this, we hypothesized that the cell can be insulated against the previously mentioned vicious cycle that leads to the opening of mPTP, and then to the disruption of ATP production.[26]

Previous studies have shown that gallic acid could improve antioxidant status through the inhibition of lipid peroxidation and that it protects the heart and lysosome membrane against isoproterenol-induced oxidative stress in rats.[29] In addition, during I/R, the administration of Polyphenol (-)-epigallocatechin gallate (EGCG) significantly reduced infarct size by 50% and the mitochondrial K_{ATP} channels played a crucial role in cardio protection induced by EGCG.[30] Also, EGCG inhibits the transcription of inducible nitric oxide synthase.[31] On the other hand, it was reported that mPTP inhibition by CsA maintained mitochondrial morphology after I/R injury in rabbits and that it limited myocyte necrosis and apoptosis.[32]

It has shown that reperfusion had some deleterious effects due to ROS production and PH disturbances during ischemia period. These phenomenon is time dependent and can led to cell death and myocardial injury which is called 'lethal reperfusion injury'.[8] In this study we had several aims. The first of our aims was to investigate the eNOS expression during early reperfusion time. Also other studies have suggested that eNOS expression increased time- dependently during the early of reperfusion (1 hour) and iNOS expression in late of it (3-24 h). This rising has some deleterious effects via NO production. The NO reacts with ROS which were produced during ischemia and formed a lot of ONOO- which was led to cell apoptosis.[33] NO has a bidirectional effects: moderate values of it has protective

effects but a high level is harmful. Second goal was to find one of mechanisms on how antioxidants can improve the hemodynamic and endothelial dysfunction via NO production and its source during early reperfusion (protective eNOS pathway).[34] So we selected one hour of reperfusion which is related to eNOS activity. Third: we measured the enzymatic activity for assessment of oxidative stress and cellular damage due to myocardial infarction in the immediately reperfusion period when typically it is at its greatest[35] and published its result which the most changes was during first 15 min of reperfusion[36] so we measured these parameters every 15 min like other studies method[35] because the I/R injury was worsened by the time and our object was to investigate the preventive role of antioxidant or mPTP inhibitor and combined effect of both during total time of experiment . We were going to check the severity of I/R injury after 60 min of reperfusion when the No production derived from eNOS was affected by pretreatment with different doses of antioxidant[37,38] only or in combination with a mPTP inhibitor (CsA). Because other studies have shown that inhibition of mitochondria permeability transition pore with this drug can prevents the eNOS expression.[39] We concluded that our antioxidant (gallic acid) modulated the I/R injury via NO production and this NO was derived from eNOS not iNOS. Investigation of hemodynamic changes during late reperfusion was not the main aim of our study.

Although having observed that the flow increased insignificantly during reperfusion in the hearts of animals pretreated by gallic acid alone, we concluded that its combination with CsA had more significant effects. The above-mentioned improvement in cardiac function, observed in our study, could be attributed to antioxidant effects of gallic acid. It seemed that scavenging of ROS by gallic acid already reported[10] could protect the cells against oxidative and nitrosative stress. In addition, by inhibition of proxy nitrite production, it could maintain the endothelium function and vascular tone. Previous studies showed that gallic acid increased eNOS expression.[2] An increase in iNOS activity and a decrease in eNOS activity during ischemia were related to I/R injuries. Our results showed that post-ischemia iNOS expression in control group increased to 1.6 times as much as that of sham group. Treatment with gallic acid or CsA alone raised eNOS/iNOS original ratio by 2 times, but the combination of both drugs was more efficient. It is because eNOS expression was increased by 3 folds, using gallic acid with CsA, but iNOS expression

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reduced to one fourth. These results corresponded to those of other researchers that suggested the increasing of eNOS/iNOS ratio with a resveratrol analogue.[40] Taken together, our results established that NO derived from eNOS probably modulated the blood flow and this effect is more efficient in the combination of gallic acid 15 mg/Kg with CsA]. In addition, eNOS expression increased by using all compounds could be attributed to enhanced bioavailability of NO during I/R injury.[41] Furthermore, our results revealed that contractility (±dp.dt[-1]), RPP, LVDP and heart rate were improved in the groups perfused by CsA in combination with gallic acid pretreatment. This effect could be ascribed to the preservation of the mitochondria against ROS by increasing the antioxidant capacity with gallic acid and through the inhibition of mPTP opening by CsA. Thus, the ATP could be kept in the cell and the Ca^{2+} overload was prevented due to the enhanced uptake of Ca^{2+} into the SR by activating the RISK pathway. Therefore, the interaction of contractile elements with each other could be improved and become faster.[42] However, using the highest dose of gallic acid (30 mg/kg) reversed these effects. According to another study, the process could be explained by a pro-oxidant effect of Gallic acid in high doses, because it has proved that ferric ion could chelate the hydroxyl group in Gallic acid molecule and reduce the oxidation potential, thereby it will lose its antioxidant activity.[43] Also, contractility was increased with CsA alone but it didn't recover with G[7.5+] CsA. This effect can be explained with I/R injury after 1 hour of reperfusion. Because 1-hour-reperfusion, contractility was decreased much more than that of 30-minute- reperfusion, so the injury became worse and the protective effect of CsA couldn't recover it to the level of 30-minute-reperfusion. On the other hand, by using higher dose of Gallic (G[15+] CsA), the antioxidant system gets stronger and prevents ROS production in mitochondria.[26] In fact, it is possible that CsA couldn't inhibit more mPTP which was opened by ROS produced due to I/R injury over the time.

Some of studies controled the heart rate with pacemaker. Also, this was a variable in our study. We were going to assess the changes of it during the experiment. So we didn't control it. Because of comparing this variable with control group, it couldn't make a bias in this study. (all groups were similar). The object was to investigate the hemodynamic parameters changes affected by I/R and pretreatment. So the heart rate was a variable that we wanted to evaluate it by it self without any intervention (using pace maker didn't satisfy this object). There are other studies using this method.[35]

Other studies showed that infarct size increased after reperfusion[21] with TTC staining. So we evaluated it during the beggining of reperfusion.

In this study, the infarct size was significantly reduced by gallic acid or CsA alone (almost 15%) but this reduction was more significant by the concomitant administration of these two drugs (almost 25%). This effect could be attributed to the activation of a protein kinases group which improved uncoupling oxidative phosphorylation and prevented the swelling of the mitochondria and cell death.[44] mPTP inhibition brought about the activation of the anti apoptotic and RISK pathways represented a mechanism for programmed cell survival. We also observed that the administration of both drugs improved the myocardial contractility more than the administration of each drug alone.

It has been already acknowledged that the CsA is not just an mPTP inhibitor. It also inhibits calcineurin which modulates mitochondrial death signaling. While other studies have reported that using FK506, a potent calcineurin inhibitor which is 100 times more potent than CsA, has not affected caspase activation. Therefore, the role of CsA in blocking the mitochondrial pores can be more important than its other roles. In addition, other studies have reported that using CsA at low doses has no toxic effects.[45] We used the lowest dose of (0.2μM) which was much lower than that of (20 mg.kg-1) used by other studies,[46] but it may be insufficient to inhibit the largest numbers of mPTP. Therefore, the effect of higher doses of the mPTP inhibitor that has laid unexplored hitherto, is highly liable to whet the appetite of interested, specialized researchers to set out further investigation of greater scrutiny.

Conclusion

In conclusion, using gallic acid as an antioxidant concomitant with the preservation of mitochondria by CsA as an mPTP inhibitor in G15+CsA in comparison with control, could have more improving effects on cardiac dysfunction, iNOS and endothelial NOS expression induced by I/R injury.

Study limitations

We had some limitations in our study, for example, our funding and facilities were very limited. So we couldn't

do more experiments. For example, we couldn't did qPCR because of funding and instruments limitation. So we forced to do the experiments using the facilities which were available. In addition, we limited to measure the protein expression. Also, we had limitation for assessment the mitochondrial function or morphology.

Ethical Approval

The protocols and procedures of the present study were approved by the animal care and use committee of the Ahvaz Jundishapur University of Medical Sciences (AJUMS.REC.1392.222).

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Author contributions

Conception and design of the research: Dianat M. Acquisition of data: Sadeghi N. Analysis and interpretation of the data: Dianat M. Statistical analysis: Sadeghi N. Obtaining financing: Badavi M. Writing of the manuscript: Sadeghi N. Critical revision of the manuscript for intellectual content: Badavi M. Análise das bandas genéticas: Samarbafzadeh A.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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