Pflugers Arch - Eur J Physiol (2009) 458:631–642
DOI 10.1007/s00424-009-0655-1

CARDIOVASCULAR PHYSIOLOGY

Green tea extract protects rats against myocardial infarction associated with left anterior descending coronary artery ligation

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Received: 7 January 2009 / Accepted: 22 February 2009 / Published online: 5 March 2009
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Abstract There is increasing evidence that green tea polyphenols can protect against myocardial damage. Recently, we showed that they bind to cardiac troponin C and alter myofilament Ca^{2+} sensitivity in cardiac muscle. In the present study, we examined whether green tea extract (GTE) could prevent the progressive remodeling seen in ischemic myocardium and improve cardiac function by modulation of the contractile apparatus utilizing a myocardial infarction (MI) model in the rat involving ligation of the left anterior descending branch. Using this model, severe myocardial injury was found, including altered cardiac performance and the appearance of extensive fibrosis and left ventricular (LV) enlargement. Supplementation with 400 mg/kg/day of GTE for 4, 18, or 46 days had beneficial effects in preventing the hemodynamic changes. Histopathological studies showed that GTE attenuated the progressive remodeling seen after myocardial injury. Echocardiography confirmed that GTE prevented LV enlargement and improved LV performance in post-MI rats. In addition, we showed that GTE supplementation for 18 or 46 days increased the myofilament Ca^{2+} sensitivity of the ischemic myocardium in post-MI rats. These results validate the novel action of green tea polyphenols in protecting against myocardial damage and enhancing cardiac contractility by modulating myofilament Ca^{2+} sensitivity in post-MI rats.

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Keywords Polyphenols · Ca^{2+} sensitivity · Cardiac troponin C · Ischemic myocardium · Echocardiography

Introduction

Green tea-derived polyphenols [e.g., catechin and (−)-epigallocatechin-3-gallate (EGCg)] have attracted much interest in the prevention of cardiovascular diseases [17, 36, 37, 39, 47]. Epidemiological studies have shown that green tea consumption is associated with reduced mortality caused by cardiovascular diseases [15, 20, 21, 31, 33]. Experimental studies have suggested that the myocardial protective effect of green tea polyphenols is associated with their antioxidant properties of scavenging reactive oxygen radicals, modulating redox-sensitive transcription factors (e.g., NFκB, AP-1), reducing the activation of signal transducers and activators of transcription (STAT)-1 and the expression of Fas receptor, and increasing NO production [2, 14, 38, 44]. More recently, green tea-derived polyphenols have been shown to have positive inotropic [16] and anti-hypertropy [13] effects, presumably via activation of the Na^+/H^+ exchanger and the reverse mode of the Na^+/Ca^{2+} exchanger [29] or a protein kinase-Cε-dependent signaling pathway [22]. However, limited information is available with regard to a direct action of green tea polyphenols on the cardiac contractile apparatus.

Ischemic heart disease is the leading cause of death worldwide. Progressive damage caused by myocardial ischemia leads to decreased contractility during heart failure, which may account for the high mortality of this disease. A surgical model of myocardial infarction (MI) associated with left anterior descending (LAD) coronary artery ligation has been widely used in rodents to study

post-ischemic ventricular remodeling [1, 10, 28, 34, 35, 43]. This surgery results in contractile dysfunction and contributes to the observed progressive thinning of the infarcted wall, ventricular enlargement, and heart failure [18]. Contractile dysfunction during ischemia is partly attributed to the effects of intracellular acidosis on the contractile apparatus [7, 30]. Intracellular acidosis and the resultant reduction in myofilament Ca^{2+} sensitivity may lead to the decreased contractility associated with myocardial ischemia [24]. This reduced Ca^{2+} sensitivity is ascribed to either a direct effect of pH on the binding of Ca^{2+} to cardiac troponin C (cTnC) [19, 26, 32] or an indirect effect via non-covalent modification of the C-terminal region of cardiac troponin I (cTnI) [6, 8, 23, 45, 46]. Our recent study showed that EGCg acts by modulating pH-induced changes in myofilament Ca^{2+} sensitivity in cardiac muscle by binding to the C-terminal sites of cTnC [25]. It was therefore of great interest to determine whether, in live animals, green tea polyphenols could improve cardiac functions by increasing myofilament Ca^{2+} sensitivity, thus overcoming the decreased contractility associated with myocardial injury.

In this study, we examined the myocardial protective effect of green tea extract (GTE) in terms of preventing progressive remodeling and improving myofilament Ca^{2+} sensitivity and thus contractile function in the ischemic myocardium utilizing the MI model in the rat with LAD ligation. Histopathological studies showed that GTE supplementation could prevent myocardial fibrosis and LV enlargement in the ischemic myocardium of post-MI animals. Echocardiography confirmed that the attenuation of the progressive remodeling of the myocardium was accompanied by improved cardiac performance in post-MI animals receiving GTE supplementation for 4, 18, or 46 days. To examine whether the myocardial protection provided by GTE supplementation involved enhancement of myofilament Ca^{2+} sensitivity, the actomyosin ATPase activity of cardiac myofibrils prepared from the hearts of 3- or 7-week post-MI rats was measured under controlled Ca^{2+} and pH conditions. This is the first study providing in vivo evidence for the myocardial protective effect of green tea polyphenols in preventing progressive remodeling in ischemic hearts and for the modulation of myofilament Ca^{2+} sensitivity in post-MI animals.

Materials and methods

Sunphenon 90DCF-T, decaffeinated green tea extract (GTE) powder was purchased from Taiyo Kagaku Co., Ltd. (Tokyo, Japan). According to the manufacturer's information, the GTE powder contained >80% polyphenols, of which >80% were catechins and >45% EGCg, and <1% caffeine. Pure EGCg was purchased from Sigma and was prepared as a 10-mM stock solution in de-ionized water. All reagents used were ACS or MB grade.

Experimental animals

Male Wistar rats (300–350 g) aged 10–11 weeks were randomly divided into three different groups, control, LAD ligation without GTE supplementation, and LAD ligation with GTE supplementation for 4, 18, or 46 days. One hundred milligrams of GTE dissolved in 0.5 ml water (2%) was administered intra-gastrically to animals (400 mg/kg animal/day). This GTE concentration used is equivalent to 180 mg EGCg/kg animal/day. The animals were housed in small groups in a temperature- (24±1°C), humidity- (55±5%), and light- (12 h light:12 h dark) controlled room until the study. During this period, the rats had access to standard rat chow and distilled water ad libitum.

Left coronary artery ligation

The LAD ligation operation is described in supplementary data. After surgery, the animals remained in intensive care until fully conscious. The immediate post-operative mortality rate of this surgical procedure was 24%. All experimental procedures conformed to the "Guidelines for Proper Conduct of Animal Experiments" approved by the Animal Care and Use Committee of National Chung-Hsing University.

Histopathological analysis

Five to six rats for each of the three groups (control, GTE, and no GTE) at the time 3 weeks and 7 weeks post-LAD ligation were studied. Rats were anesthetized with ketamine (80 mg/kg IP) and the heart removed, washed with saline for 10 min, and fixed in neutral-buffered 4% formalin for 48 h. Paraffin-embedded samples were sectioned (4 μm thick) and stained with hematoxylin and eosin (H&E staining) to assess the overall morphology [11].

To delineate infarct size, hearts were sectioned from the apex to the base into five slices. Each slice (3 mm thick) was incubated for 10 min at 32°C in 1% triphenyltetrazolium chloride (TTC, Alfa Aesar, Ward Hill, MA, USA) in PBS, pH 7.4. The non-infarcted myocardium was stained red, while the infarcted myocardium appeared white. The slices were photographed using a digital camera and the images analyzed using the computerized Image-Pro Plus software (Media Cybernetics Inc, Silver Spring, MD, USA). Infarct size was expressed as a percentage of the mass of the whole myocardium [49].

Masson trichrome staining was performed to delineate the area of fibrosis in the myocardium [5]. Photographs

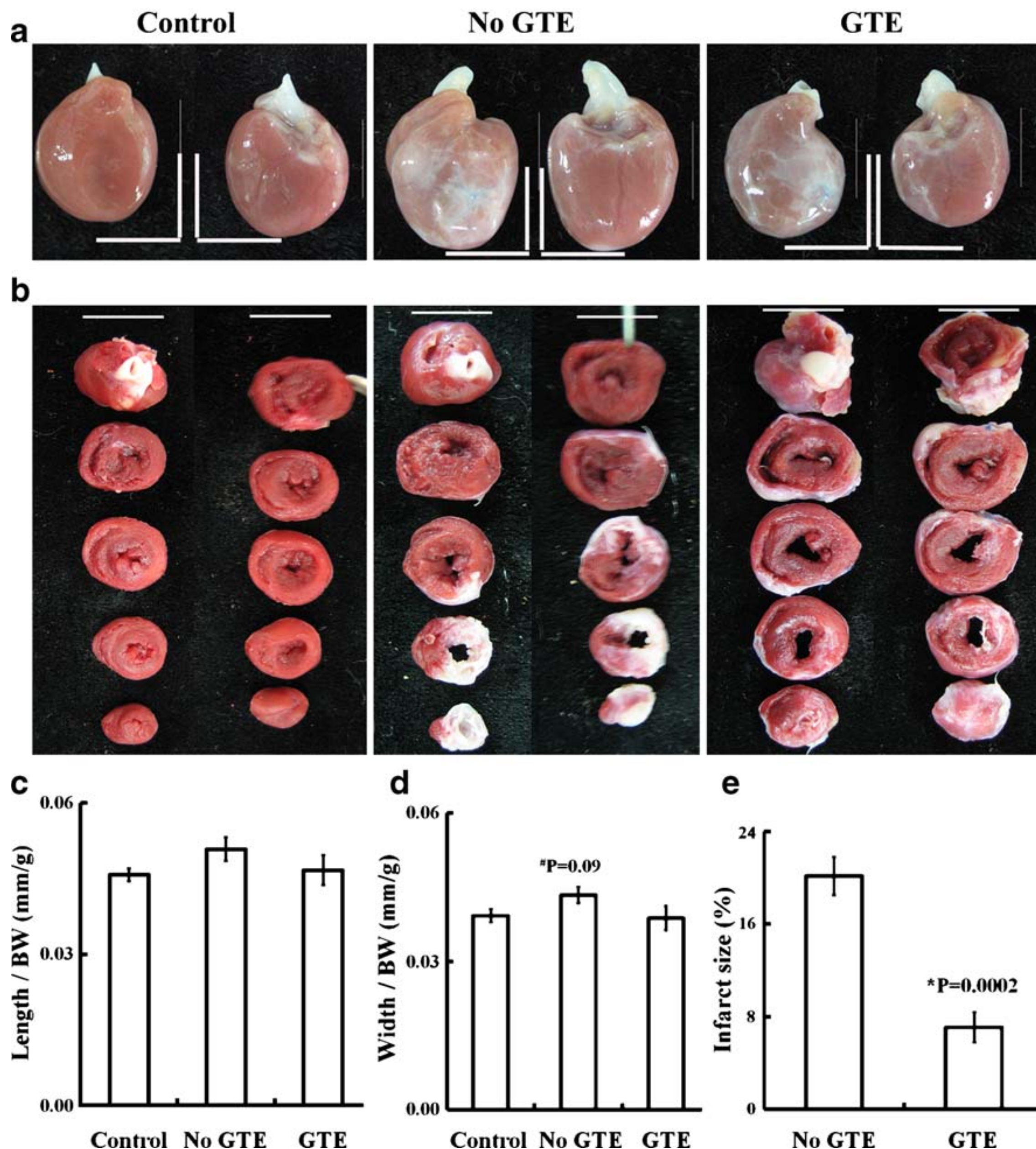
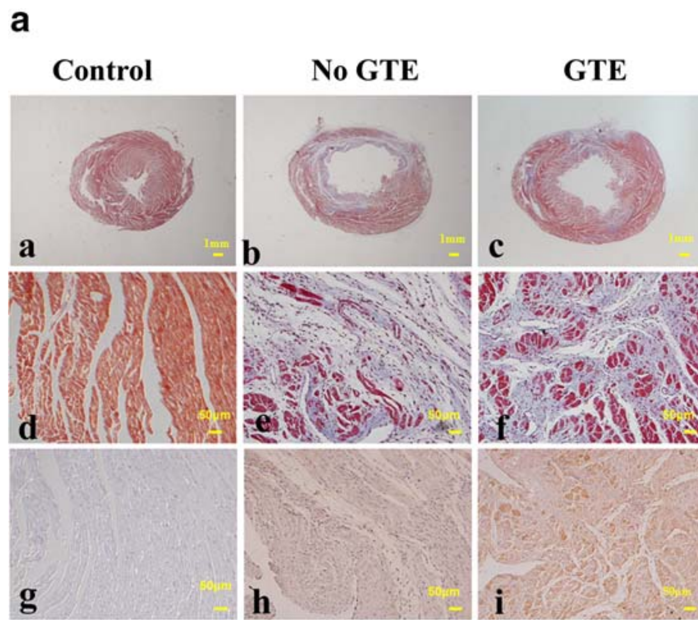
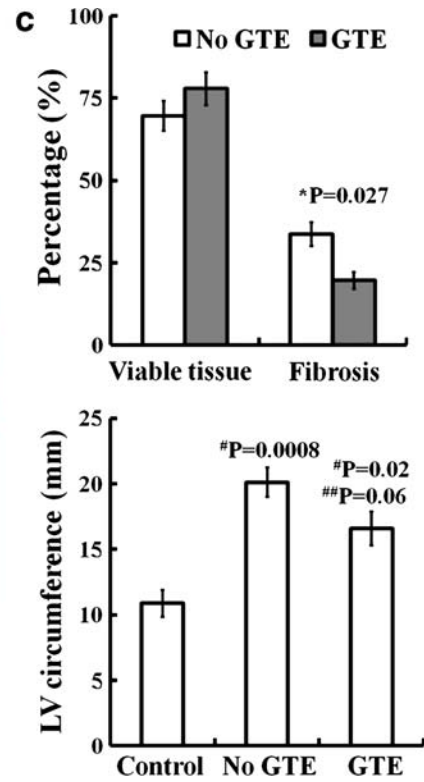


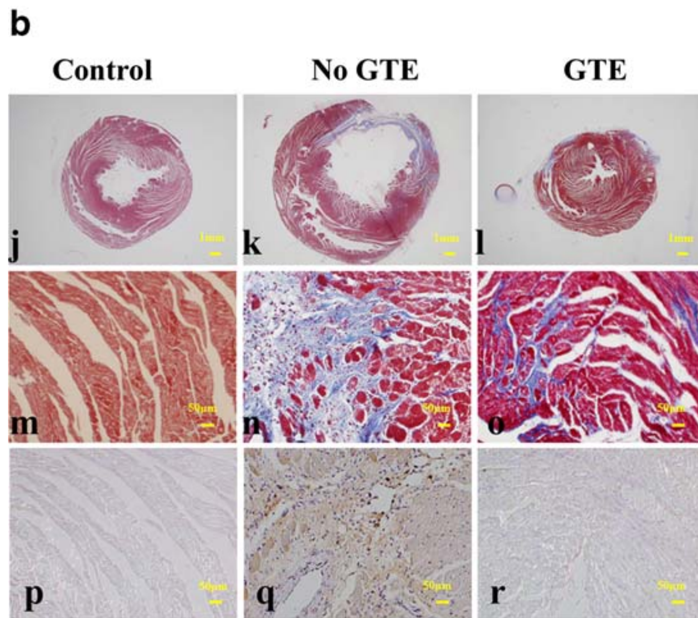
Fig. 1 GTE supplementation protects against myocardial injury in rats at 7 weeks post-MI. **a** Morphological changes in the heart associated with myocardial injury with and without GTE supplementation. The calibration bar is 1 cm. **b** TTC staining of tissue slices from controls or at 7 weeks post-MI with and without GTE supplementation. To determine infarct size, the heart was sectioned from the apex to the base into five slices which were stained with TTC. Non-infarcted myocardium is stained red, while infarcted myocardium is white. The calibration bar is 1 cm. **c**, **d** Quantitative analysis of the length (**c**) and width (**d**) normalized to body weight in controls ($n=5$) and MI rats with ($n=9$) and without ($n=8$) GTE treatment. Each value is the mean±SEM. *$P<0.1$ between control and MI rats without GTE treatment. **e** Infarct size in the hearts of MI rats with ($n=7$) and without GTE ($n=5$) supplementation. Infarct size was determined as described in the Methods and expressed as a percentage of the mass of the whole heart. Each value is the mean±SEM. *$P<0.05$ between MI rats with and without GTE treatment

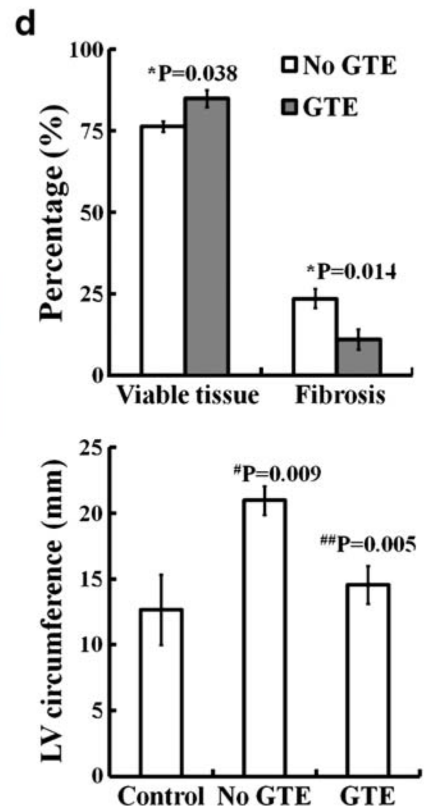
were obtained using an Olympus SZX7 stereomicroscope and inverted microscope (Olympus Co., Tokyo, Japan). The slides were observed on a stereomicroscope with the whole field view (8×) and three non-contiguous slides per rat were randomly chosen to calculate the fibrotic area (blue) and viable area (red) normalized to the area of the whole heart using Image-Pro Plus computerized software (Media Cybernetics Inc). The largest endocardial circumference measured in the three LV sections was used as the index for the extent of LV enlargement.

Histoimmunocytochemistry was performed to detect osteopontin (OPN) expression in the myocardium. The paraffin-









embedded hearts were sectioned (4 µm thick), the sections deparaffinized, and endogenous peroxidase blocked with 0.3% hydrogen peroxide for 10 min. After washing with PBS, nonspecific binding was minimized by blocking with 1% normal horse serum for 1 h and the sections washed with PBS and incubated overnight at 4°C with rabbit antiserum (1:200) against porcine OPN produced by a local biotechnology company (Ig Medica Biotechnology, Taiwan) using recombi-

◀ **Fig. 2** Histopathological analysis. **a**, **b** Masson trichrome staining and OPN histoimmunocytochemistry of control (*a*, *d*, *g*, *j*, *m*, *p*) and post-MI rats at 3 weeks (**a**) or 7 weeks (**b**) with (*c*, *f*, *i*, *k*, *n*, *q*) or without (*b*, *e*, *h*, *l*, *o*, *r*) GTE supplementation. *Top row*: photographs were obtained using a stereomicroscope (×8) for Masson trichrome staining of the myocardium. The calibration bar indicates 1 mm. The fibrotic area is stained blue and the viable area red. *Center row*: Photographs were taken using an inverted microscope (×200) for Masson trichrome staining of the myocardium. The calibration bar represents 50 μm. *Bottom row*: photographs of the histoimmunocytochemical detection of OPN in the myocardium. **c**, **d** Quantitative analysis of the effects of GTE on myocardial fibrosis and endocardial circumference in 3- (**c**) or 7 (**d**) week post-MI rats. *Upper panels*: the percentage of the total area that was fibrotic or viable was estimated in MI rats with or without GTE supplementation as described in the Methods. *Lower panels*: the LV circumference was estimated in control and post-MI rats with and without GTE supplementation as described in the Methods. Each value is the mean±SEM for measurements taken from five to six animals. *Significant difference ($P<0.05$) between control and MI rats with and without GTE treatment. #Significant difference ($P<0.05$) between control and MI rats with or without GTE treatment. ##Significant difference ($P<0.05$) between MI rats with and without GTE treatment

nant porcine OPN protein purified in our laboratory. The sections were then incubated with a biotin-conjugated goat anti-rabbit IgG secondary antibody (1:600, ABC kit, Vector Inc.), as recommended by the manufacturer. After the enzymatic reaction of horseradish peroxidase (HRP) with diaminobenzidine (DAB), OPN-containing tissues were stained brown [5].

Echocardiography

Rats were anesthetized with intraperitoneal ketamine (80 mg/kg) and echocardiographic images taken with a 7.5-MHz probe (Hewlett-Packard, MA, USA). The diastolic inter-ventricular septum thickness (IVSd), diastolic left ventricular inner diameter (LVIDd), end diastolic left ventricular thickness (EDLV), and systolic LV inner diameter (LVIDs) were measured by M-mode echocardiography in the control group and post-MI groups with or without GT supplementation for 4, 18, or 46 days. The LV fractional shortening (FS) and LV ejection fraction (EF) were determined using the equations:

$$FS(\%) = (LVIDd - LVIDs)/LVIDd * 100 \quad (1)$$

$$EF(\%) = \left(LVIDd^2 - LVIDs^2\right)/LVIDd^2 * 100 \quad (2)$$

ATPase activity assay

In this experiment, we have four groups of rats for study: control with and without GTE supplementation and MI animals with and without GTE supplementation. Myofibrils were prepared from the left ventricular muscle of four different groups of rats: (1) controls without GTE supplementation, (2) controls with GTE supplementation for 18 or 46 days, (3) 3 or 7 weeks post-LAD ligation without GTE supplementation, and (4) LAD ligation with GTE supplementation for 18 or 46 days, starting 3 days after LAD ligation, according to previously described procedures [27]. Myofibrillar ATPase activity was determined in the presence and absence of 0.1 mM EGCg by measuring inorganic phosphate release. The relationship between myofibrillar ATPase activity (nmol Pi/min/mg protein) and the pCa was determined using the Hill plot as described previously [25]. From the plot, the Hill coefficient (n), as a measure of cooperativity, and the Ca^{2+} concentration giving half-maximal activation ($pCa_{1/2}$) were obtained for the Ca^{2+}-activated myofibrillar ATPase activity.

Statistics

Quantitative values were expressed as the mean±SEM. An unpaired two-tailed Student's t test and one-way ANOVA were performed for between-group comparisons. Scheffe's multiple range test was used following ANOVA to determine which groups differed from each other. For all tests, P values less than 0.05 were considered as significant.

Results

In this study, GTE was given daily to the test rats from day 3 after ligation, so the terms day 4, 18, and 46 of GTE treatment and weeks 1, 3, and 7 after ligation are equivalent. Figure 1 shows morphological changes in the heart associated with myocardial injury in the rats at 7 weeks post-MI. The hearts of the post-MI rats ("no GTE") showed an increase in width (Fig. 1a, d), but not in length (Fig. 1a, c), and GTE supplementation for 46 days ("GTE") restored the width back to control levels (Fig. 1a, c). Note that, both with and without GTE supplementation, the surface of the heart of the post-MI rats was covered with whitish fibrotic tissue (Fig. 1a). However, TTC staining showed an infarct size of 20.2±1.7% of the whole heart mass in post-MI rats without GTE supplementation, but only 7.1±1.3% in post-MI rats with GTE supplementation for 46 days (Fig. 1b, e). This shows that GTE administration attenuated myocardial infarction (↓65%) in post-MI animals.

To further examine the effect of GTE supplementation on the progression of myocardial remodeling in post-MI rats, Masson trichrome staining for detection of fibrosis and histoimmunocytochemical detection of osteopontin (OPN) in the myocardium were performed. As shown in Fig. 2a

and c, post-MI rats at 3 weeks showed myocardial fibrosis (24.1±2.6%) associated with OPN expression in the myocardium and LV enlargement (LV circumference= control, 10.9±1.9 mm; MI, 20.1±1.1 mm; *P=0.0008), and GTE supplementation for 18 days resulted in a significant 40% reduction in cardiac fibrosis (14.5±2.6%) and a significant 17% reduction in LV enlargement (LV circumference=16.6±1.3 mm). The effects of GTE on myocardial fibrosis and LV enlargement were even more obvious at 7 weeks post-MI (Fig. 2b, d). Extensive interstitial fibrosis and LV enlargement were still seen in the myocardium of rats at 7 weeks post-MI (fibrosis= 19.0±2.4%; LV circumference=21.0±1.0 mm), and GTE supplementation for 46 days resulted in a significant reduction in cardiac fibrosis (68%) and LV enlargement (30%) (fibrosis=6.1±1.0%; LV circumference=14.6± 1.4 mm). Note that GTE supplementation for 18 days had a non-significant effect (P=0.18) on the viable area of the myocardium in MI rats, whereas supplementation for 46 days resulted in a significant increase (↑8%, *P=0.038) (Fig. 2c, d).

Echocardiography was performed to assess LV dimensions and function. Figure 5 shows representative M-mode echocardiographic recordings (Fig. 3a) and average data for LV dimensions and function (Fig. 3b) in controls and post-MI rats with and without GTE supplementation for 4, 18, or 46 days. The diastolic inter-ventricular septum thickness (IVSd) was significantly greater (↑23%) in control rats (2.2±0.1 mm, n=5) than in post-MI rats (1.7±0.1 mm, n=12), while the diastolic LV inner diameter (LVIDd) was larger (↑25%) in post-MI rats (7.4±0.1 mm, n=12) than in controls (5.9±0.1 mm, n=5). Consistent with the histopathological data, the echocardiographic data indicated that the myocardial septum became thinner and the LV enlarged in post-MI rats. In addition, the contractile performance of the LV evaluated by the ejection fraction (*EF %*=control, 77.9±2.6%; MI, 51.0±5.3%) and fractional shortening (*FS %*=control, 53.3±2.9%; MI, 30.1±43%) was significantly reduced in post-MI rats (↓35% *EF* and↓43% *FS*). After GTE supplementation for 4, 18, or 46 days, the LVIDd was significantly reduced in post-MI rats (9% at day4, n=6; 19% at day18, n=9; 19% at day46, n=7) and the IVSd significantly increased (11% at day4, n=6; 20% at day18, n=9; 29% at day46, n=7). The calculated *EF %* (31% at day4, 55% at day18, 73% at day46) and *FS %* (38% at day4, 50% at day18, 95% at day46) for LV contractile performance were also increased by GTE supplementation. These results suggest that GTE supplementation prevents progressive remodeling of the myocardium and restores LV contractile performance.

To understand the mechanism of the GTE-induced improvement in myocardial performance, skinned cardiac myofibrils prepared from the hearts of control and post-MI rats with or without GTE supplementation for 18 or 46 days were tested for Ca^{2+}-dependent actomyosin ATPase activity in the presence and absence of 0.1 mM EGCg (Fig. 4, Tables 1 and 2). As shown in Fig. 4a, when Ca^{2+}-dependent myofibrillar ATPase activity was measured in the absence of EGCg, the Ca^{2+} sensitivity was not significantly different in control rats with (6.29±0.09, n=6) or without (6.28±0.07, n=6) GTE supplementation for 18 days. In contrast, rats at 3 weeks post-MI displayed depressed myofilament Ca^{2+} sensitivity, as shown by a right-shift of the plot of pCa vs. myofibrillar ATPase activity ($pCa_{1/2}$=5.80±0.07, n=6), and GTE supplementation for 18 days restored myofilament Ca^{2+} sensitivity ($pCa_{1/2}$=6.18±0.13, n=6) to controls levels (top panel). In contrast, when the assay was performed in the presence of 0.1 mM EGCg, the controls with GTE supplementation for 18 days ($pCa_{1/2}$=6.33±0.03, n=6) showed a left-shift in Ca^{2+} sensitivity compared to those without GTE supplementation ($pCa_{1/2}$=5.70±0.03, n=6) (bottom panel). This result confirmed our previous finding that 0.1 mM EGCg causes a decrease in myofilament Ca^{2+} sensitivity in the porcine cardiac myofibrillar ATPase assay [25]. In the presence of 0.1 mM EGCg, myofilament Ca^{2+} sensitivity was not significantly different in cardiac myofibrils isolated from the hearts of post-MI rats with ($pCa_{1/2}$= 6.21±0.03, n=6) or without GTE supplementation ($pCa_{1/2}$= 6.16±0.03, n=6). Similar results were seen in rats at 7 weeks post-MI (Fig. 4b).

Intracellular acidosis is an important factor in the reduced contractility associated with myocardial ischemia. Consistent with our previous report [25], 0.1 mM EGCg caused a significant reduction in the acidic pH-induced decrease in Ca^{2+}-dependent myofibrillar ATPase activity in control rats without GTE supplementation (top panel in Fig. 5a and b). In control rats supplemented with GTE for 18 or 46 days (second panel from top in Fig. 5a or b, respectively), 0.1 mM EGCg did not significantly affect the Ca^{2+} sensitivity at pH 7.0 or 6.5, but significantly increased the sensitivity at pH 6.0 ($pCa_{1/2}$ ↑0.24 at 18 days; ↑0.34 at 46 days). In post-MI rats without GTE supplementation for 3 or 7 weeks (third panel from top in Fig. 5a or b, respectively), 0.1 mM EGCg caused a significant increase in Ca^{2+} sensitivity at pH 7 ($pCa_{1/2}$ at 3 weeks=↑0.36; 7 weeks=↑0.43), pH 6.5 (3 weeks=↑0.26; 7 weeks=↑0.19), and pH 6.0 (3 weeks=↑0.38; 7 weeks=↑0.25). However, in post-MI rats with GTE supplementation for 18 or 46 days (bottom panel in Fig. 5a or b, respectively), the effects of EGCg on myofilament Ca^{2+} sensitivity were generally reduced over the pH range of 7 to 6. The summarized Ca^{2+} sensitivity ($pCa_{1/2}$) and Hill coefficient (n) for the Ca^{2+}-dependent myofibrillar ATPase activity measured in the presence and absence of 0.1 mM EGCg at pH 7.0, 6.5, and 6.0 for 3- and 7-week post-MI rats are listed in Tables 1 and 2, respectively. Clearly, green tea polyphenols modulated

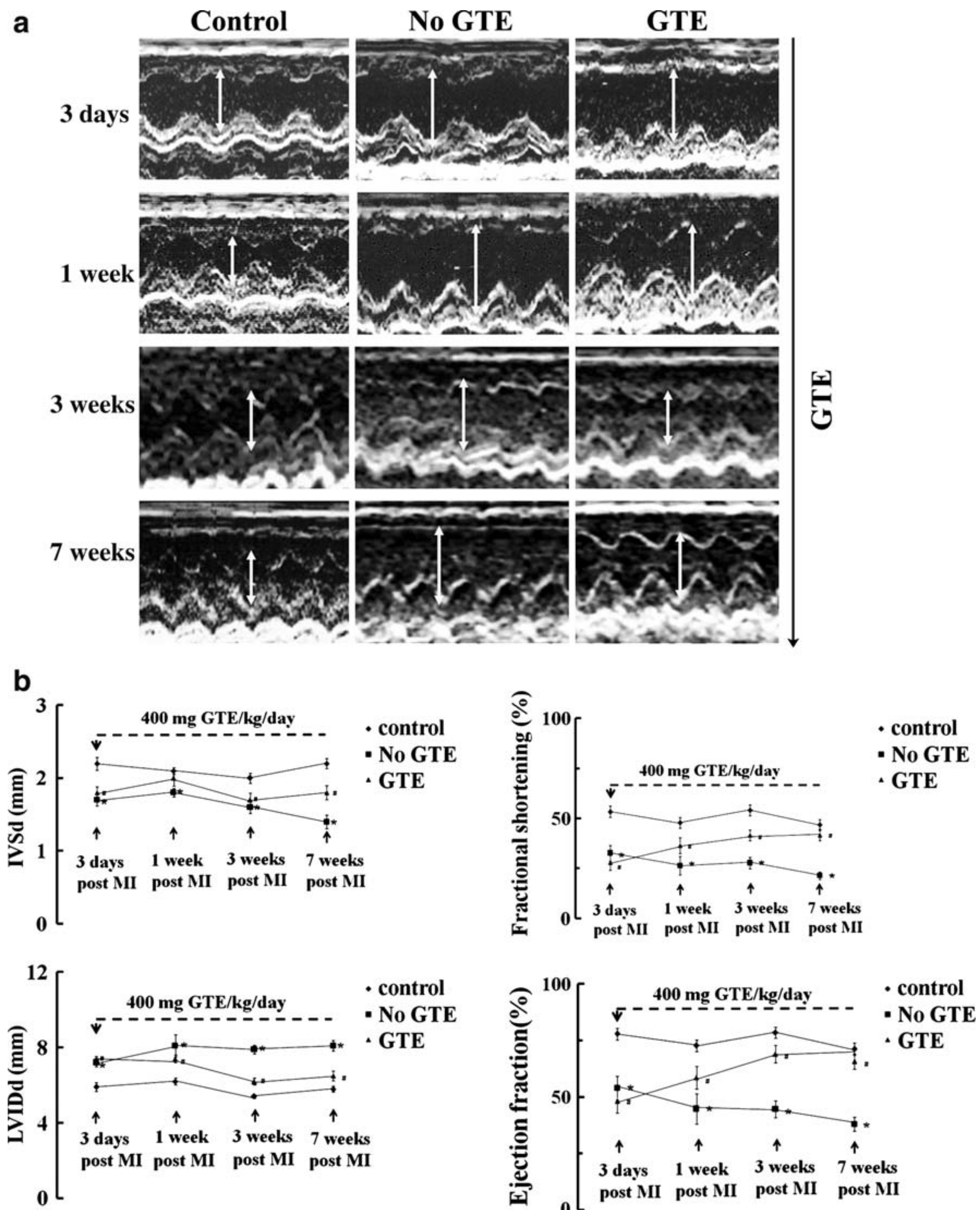


Fig. 3 Echocardiography. **a** Representative M-mode echocardiographic recordings and **b** average data for LV dimensions and function in controls and post-MI rats with and without GTE supplement for 4, 18, or 46 days. The diastolic inter-ventricular septum thickness (IVSd, *left upper panel*), diastolic left ventricular inner diameter (LVIDd, *left lower panel*), end diastolic left ventricular thickness (EDLV), and systolic LV inner diameter (LVIDs) were measured by M-mode echocardiography. LV fractional shortening (FS, *right upper panel*) and LV ejection fraction (EF, *right lower panel*) were determined as described in the Materials and methods. Each value is the mean±SEM with measurements from six to seven animals. *Significant difference ($P<0.05$) between control and MI rats with or without GTE treatment

myofilament Ca^{2+} sensitivity in the myocardium in the animal model, while EGCg potentiated myofilament Ca^{2+} sensitivity for myocardial contractility under acidic conditions in vitro.

Discussion

In contrast to the well-known effects of green tea on vessels, little information is available regarding its myocar-

Table 1 Myofilament Ca^{2+} sensitivity by measurement of ATPase activity

	pH 7.0		pH 6.5		pH 6.0	
	$pCa_{1/2}$	*n*	$pCa_{1/2}$	*n*	$pCa_{1/2}$	*n*
No EGCg						
Control	6.28±0.07	1.23±0.08	5.57±0 .01	1.99±0.09	4.81±0.06	1.58±0.14
Control with GTE supplementation	6.29±0.09	*1.55±0.13	5.53±0.11	1.97±0.45	*5.10±0.07	1.79±0.31
MI	*5.80±0.07	1.54±0.29	*5.28±0.09	*1.36±0.16	*4.59±0.01	1.51±0.11
MI with GTE supplementation	6.18±0.13	1.60±0.19	5.61±0.09	1.69±0.22	*4.97±0.04	*2.38±0.29
0.1 mM EGCg						
Control	5.70±0.03	2.48±0.12	5.45±0.05	1.65±0.24	5.01±0.07	1.87±0.24
Control with GTE supplementation	*6.33±0.03	*1.34±0.08	*5.68±0.05	*2.0±0.08	*5.34±0.03	*2.98±0.15
MI	*6.16±0.03	*1.85±0.17	5.54±0.06	1.64±0.15	4.97±0.06	1.61±0.25
MI with GTE supplementation	*6.21±0.03	*1.49±0.09	*5.81±0.05	1.63±0.23	4.88±0.07	1.39±0.11

Hill coefficient (*n*) and the Ca^{2+} concentration giving half-maximal activation ($pCa_{1/2}$) in cardiac myofibrils from control and post-MI rats with or without GTE supplementation for 18 days. Myofibrillar ATPase activity was measured by suspending myofibrils in buffer at pH 7.0, pH 6.5, or pH 6.0 in the presence or absence of 0.1 mM EGCg, as described in Fig. 5. Each value is the mean±SEM for five measurements. * symbolizes the significant difference of a Student's *t* test between control rats without GTE and (1) control rats with GTE supplementation, (2) post-MI rats with GTE supplementation, and (3) post-MI rats without GTE supplementation , where *P* values less than 0.05

dial effects. Using an experimental autoimmune myocarditis model in rats, Suzuki et al. showed that green tea catechins reduced inflammation and suppressed ventricular remodeling [40]. The same research teams utilizing another animal model of chronic myocardial ischemia in the rats with LAD ligation demonstrated that catechins attenuated chronic ventricular remodeling after myocardial ischemia due to the suppression of proinflammatory factors without systemic adverse effects [41]. A recent study with neonatal rat cardiomyocytes showed the cardioprotective effects of EGCg on H_2O_2-mediated oxidative stress via upregulation of antioxidative enzyme (e.g., heme oxygenase-1; HO-1) and activation of prosurvival signaling kinases (e.g., Akt, $ERK_{1/2}$, p38 MAPK) [9]. Since inhibition of HO-1 and these signaling kinases does not abolish polyphenol-mediated protection, upregulation of antioxidative enzyme and activation of prosurvival signaling do not appear to play a major role in polyphenol-mediated cardioprotection.

One of the novel features of the present study was the use of a surgical model of MI in rats with LAD ligation to evaluate the myocardial protective effect of GTE. In this animal model, severe myocardial injury, including altered

Table 2 Myofilament Ca^{2+} sensitivity by measurement of ATPase activity

	pH 7.0		pH 6.5		pH 6.0	
	$pCa_{1/2}$	*n*	$pCa_{1/2}$	*n*	$pCa_{1/2}$	*n*
No EGCg						
Control	6.22±0.04	1.65±0.16	5.52±0.05	1.57±0.24	4.81±0.054	1.58±0.16
Control with GTE supplementation	6.18±0.10	1.35±0.14	5.52±0.06	*2.19±0.12	4.89±0.06	1.95±0.25
MI	*5.61±0.12	1.55±0.25	*5.20±0.05	1.30±0.11	*4.53±0.07	*1.22±0.07
MI with GTE supplementation	*5.84±0.05	1.50±0.17	5.48±0.01	1.24±0.12	4.86±0.04	1.36±0.14
0.1 mM EGCg						
Control	5.73±0.09	1.73±0.27	5.54±0.05	1.47±0.24	5.05±0.03	1.85±0.28
Control with GTE supplementation	*6.23±0.07	1.74±0.26	5.50±0.13	1.31±0.13	5.23±0.14	*1.10±0.08
MI	*6.04±0.06	1.68±0.33	5.41±0.13	1.63±0.20	4.78±0.19	1.85±0.18
MI with GTE supplementation	*6.10±0.09	1.47±0.20	5.56±0.07	1.73±0.30	4.98±0.16	1.33±0.15

Hill coefficient (*n*) and the Ca^{2+} concentration giving half-maximal activation ($pCa_{1/2}$) in cardiac myofibrils from control and post-MI rats with and without GTE supplementation for 46 days. * symbolizes the significant difference between control rats without GTE and (1) control rats with GTE supplementation, (2) post-MI rats with GTE supplementation, and (3) post-MI rats without GTE supplementation, where *P* values less than 0.05. The details are identical to those in Table 1

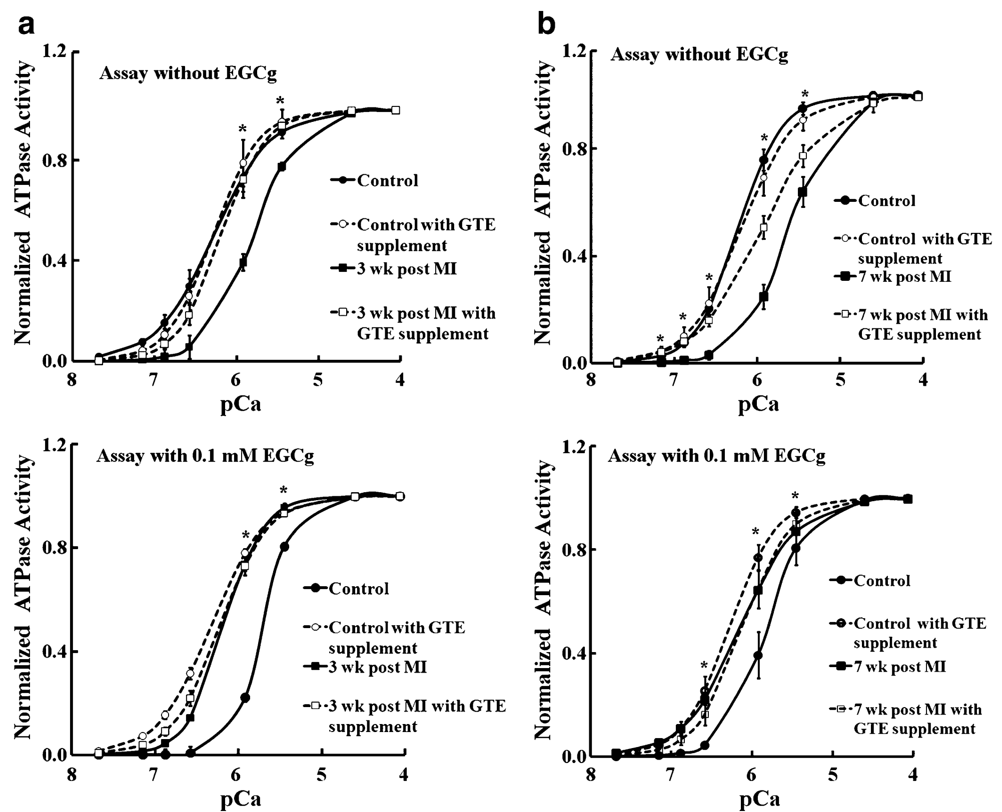


Fig. 4 Measurement of Ca^{2+}-dependent myofibrillar ATPase activity. Cardiac myofibrils prepared from the hearts of control and post-MI rats with and without GTE supplementation for 18 (**a**) or 46 (**b**) days. Acto-myosin ATPase activity was measured in the absence (*upper panel*) or presence (*lower panel*) of 0.1 mM EGCg as described in the Methods. The measured ATPase activity at pCa 8 was subtracted from that at the test pCa and the value normalized to that at pCa 4 and plotted against the pCa. Each value is the mean±SEM for five measurements. * symbolizes the significant difference ($P<0.05$) for one-way ANOVA among four different groups: (1) controls without GTE supplementation, (2) controls with GTE supplementation, (3) LAD ligation without GTE supplementation, and (4) LAD ligation with GTE supplementation

cardiac performance and the appearance of extensive fibrosis and LV enlargement, was seen. Histopathological studies showed that GTE supplementation for 18 or 46 days attenuated progressive remodeling after myocardial injury (Figs. 1 and 2). Transthoracic echocardiography confirmed that GTE supplementation prevented LV enlargement and improved LV performance in post-MI rats (Fig. 3). The data reported here validate the novel action of green tea polyphenols in protecting post-MI rats against myocardial damage and enhancing cardiac contractility.

Troponin (Tn) is a myofilament switch which directly controls the Ca^{2+}-dependent activation of striated muscle contraction [12]. The Tn complex contains three subunits, troponin C (TnC), the Ca^{2+}-binding inhibitory subunit troponin I (TnI), and troponin T (TnT), which binds the complex to tropomyosin. Phosphorylation of cTnI and cTnT mediated by protein kinases A and C can modulate myofilament Ca^{2+} sensitivity and cross-bridge cycling in the myocardium [7, 30]. Covalent modification of cTnI and cTnT is thought to play an essential role in tuning myocardium performance in both normal and diseased hearts [7, 19, 24, 30]. Accordingly, our study showing that post-MI rats displayed depressed myofilament Ca^{2+} sensitivity of cardiac myofibrils could be attributed to the modification of these myofilament proteins (Fig. 4).

A recent study showed that mutation of Gly159 of cTnC to Asp reduces the myofilament desensitization induced by phosphorylation of Ser23 and Ser24 of cTnI by altering Ca^{2+} binding to cTnC and that a site made up of the N-terminal region of cTnI, the C-lobe of cTnC, and the C-terminus of cTnT is important in modulating myofilament Ca^{2+} sensitivity in cardiac muscle [3]. More recently, studies with NMR spectroscopy [42] and biochemical approaches [25] have shown that EGCg binds to the C-lobe of cTnC near the region involved in the interaction with the N-terminal helix of cTnI and that this might alter Ca^{2+} binding to the C-terminal Ca^{2+}-binding sites. By disrupting and weakening the interaction of $cTnI_{34–71}$ with the cTnC C-lobe in myofilaments, EGCg has compensatory effects on increasing Ca^{2+} sensitivity of cardiac myofibrils in post-MI rats (Fig. 4).

Non-covalent modification of cardiac Tn also occurs, e.g., the C-terminal region of cTnI (residues 153–164) is proton-sensitive and non-covalent modification occurs on intracellular acidification [6, 8, 23, 26, 32, 45, 46]. Recently, we showed that EGCg binding to the C-lobe of cTnC reduces the acidic pH-induced decrease in Ca^{2+} sensitivity [25]. Based on circular dichroism (CD) and intrinsic fluorescence spectroscopy measurements, the K_d for the binding of EGCg to cTnC was estimated as 3–4 μM. The binding of EGCg to cTnC was found to be Ca^{2+} independent, but dependent on the proton concentration. The interaction between EGCg and cTnC was twice as strong at pH 7 as at pH 6.5 [25]. In the present study, we confirmed further that green tea polyphenols potentiate myofilament Ca^{2+} sensitivity of cardiac myofibrils prepared

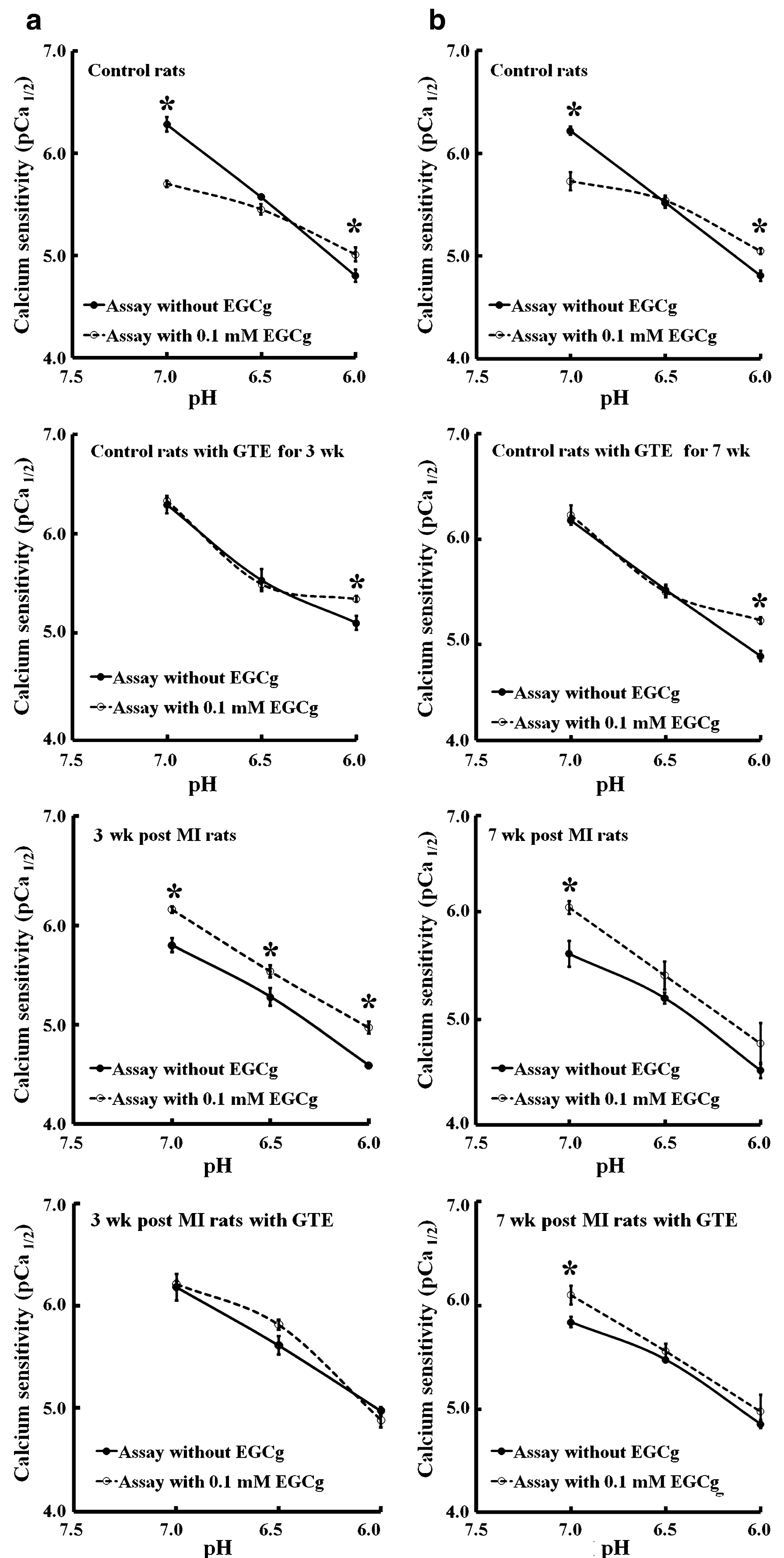


Fig. 5 Quantitative analysis of the effects of EGCg on the pH-induced decrease in the Ca^{2+} sensitivity ($pCa_{1/2}$) of myofibrillar ATPase activity. Control and post-MI rats with and without GTE supplementation for 18 (**a**) or 46 (**b**) days were compared. Actomyosin ATPase activity was measured in the presence (*dotted lines*) or absence (*solid lines*) of 0.1 mM EGCg. The normalized ATPase activity is plotted against the pCa. Each value is the mean± SEM for five measurements. * symbolizes the significant difference ($P<0.05$) of a Student's *t* test between measurements of Ca^{2+}-dependent actomyosin ATPase activity in the presence and absence of 0.1 mM EGCg

from the hearts of both control and post-MI animals under acidic conditions (Fig. 5).

There are some studies indicating that a patient needs to drink at least 1 l of tea (about four cups)/day to gain some degree of benefit after a myocardial infarction [14, 33]. A cup of green tea contains 20–100 mg EGCg. According to the process of extraction of green tea reported by Yang et al., green tea leaves were first decaffeinated using supercritical carbon dioxide, followed by extraction with boiling water and lyophilization [48]. This decaffeinated GTE powder, containing 7.3% EGCg and 11.5% other catechins (e.g., ECG, EGC, EC), was used to study bioavailability of green tea polyphenols in human [48]. Their measurements indicated that the maximal plasma concentration of EGCg (0.3–1.1 μg/ml) was observed at 1.4–2.4 h after GTE ingestion. The half-life of EGCg and other catechins was 5.0–5.5 h and 2.5–3.4 h, respectively. A similar study with bioavailability of green tea polyphenols in rats was also conducted by Chen et al. [4] to investigate the absorption, distribution, and elimination of EGCg, and catechins after administration of GTE (200 mg or 15 mg EGCg equivalent/kg animal) or pure EGCg (75 mg/kg animal). Their data showed that the maximal plasma concentration of EGCg (16.3 ng/ml) appeared at 1.24 h after GTE ingestion. The elimination half-life of EGCg was 3.5 h after GTE ingestion. However, EGCg displayed different pharmacokinetic behavior when EGCg was given in the GTE or pure EGCg. In comparison with GTE supplementation, pure EGCg given to animals showed a 3.6-fold lower absorption rate constant, a 6.9-fold smaller distribution, and a 1.6-fold faster elimination of EGCg, respectively. This different pharmacokinetic behavior might be due to complex formation between EGCg and other components in GTE [4]. In our study, we showed that GTE supplementation (400 mg/kg animal/day, equivalent to180 mg EGCg/kg animal/day) for 18 or 46 days attenuated progressive remodeling after myocardial injury, prevented LV enlargement, and improved LV performance in post-MI rats. Apparently, the GTE concentration used in this study is relatively high. We do not rule out the possibility that a high dose of GTE will trigger additional protective signal transduction pathways in cardiomyocytes. It should be useful to determine the concentration-dependent effects of GTE or pure EGCg on myocardial protection/recovery from MI.

In summary, our results show that green tea polyphenols protect against myocardial damage and enhance cardiac contractility by modulating myofilament Ca^{2+} sensitivity in post-MI rats.

Acknowledgments This work was supported in part by the National Science Council of Taiwan (NSC 95-2320-B-005-005) and cooperative projects between the Taichung Veterans General Hospital and the NCHU (TCVGH-NCHU 967602).

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