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An Oral Formulation of Angiotensin-(1-7) Produces Cardioprotective Effects in Infarcted and Isoproterenol-Treated Rats

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Abstract—In this study we evaluated the cardiac effects of a pharmaceutical formulation developed by including angiotensin (Ang)-(1-7) in hydroxypropyl β -cyclodextrin (HP β CD), in normal, infarcted, and isoproterenol-treated rats. Myocardial infarction was produced by left coronary artery occlusion. Isoproterenol (2 mg/kg, IP) was administered daily for 7 days. Oral administration of HP β CD/Ang-(1-7) started immediately before infarction or associated with the first dose of isoproterenol. After 7 days of treatment, the rats were euthanized, and the Langendorff technique was used to analyze cardiac function. In addition, heart function was chronically (15, 30, 50 days) analyzed by echocardiography. Cardiac sections were stained with hematoxylin/eosin and Masson trichrome to evaluate cardiac hypertrophy and damage, respectively. Pharmacokinetic studies showed that oral HP β CD/Ang-(1-7) administration significantly increased Ang-(1-7) on plasma whereas with the free peptide it was without effect. Oral administration of HP β CD/Ang-(1-7) (30 μ g/kg) significantly reduced the deleterious effects induced by myocardial infarction on systolic and diastolic tension, \pm dT/dt, perfusion pressure, and heart rate. Strikingly, a 50% reduction of the infarcted area was observed in HP β CD/Ang-(1-7)-treated rats. Furthermore, HP β CD/Ang-(1-7) attenuated the heart function impairment and cardiac remodeling induced by isoproterenol. In infarcted rats chronically treated with HP β CD/Ang-(1-7), the reduction of ejection fraction and fractional shorting and the increase in systolic and diastolic left ventricular volumes observed in infarcted rats were attenuated. Altogether, these findings further confirm the cardioprotective effects of Ang-(1-7). More importantly, our data indicate that the HP β CD/Ang-(1-7) is a feasible formulation for oral administration of Ang-(1-7), which can be used as a cardioprotective drug. (*Hypertension*. 2011;57:477-483.) • **Online Data Supplement**

Key Words: angiotensin ■ drugs ■ myocardial infarction ■ heart failure ■ hypertrophy

Angiotensin (Ang)-(1-7) is a biologically active peptide of the renin-angiotensin system, of which the actions are often opposite to those attributed to Ang II. This fact, in addition to the Ang-(1-7) ability to inhibit the angiotensin-converting enzyme (ACE)¹ and to the increase in its plasma levels after administration of drugs that block the renin-angiotensin system activity,² suggests that this peptide could represent a target to develop novel and innovative cardioprotective drugs. Indeed, several studies have demonstrated that this heptapeptide exerts important beneficial effects in the heart, such as amplification of the coronary dilation induced by bradykinin,^{3,4} reduction of release of norepinephrine,⁵ improvement in cardiac function,⁶⁻⁹ and regulation of cardiac remodeling and cell growth.¹⁰⁻¹⁴ These effects are generally mediated by the activation of the G protein-coupled receptor

Mas,^{3,8,13,15} which was identified as an endogenous binding site for Ang-(1-7).¹⁶ Thus, Ang-(1-7), in conjunction with its receptor Mas and ACE2, the main enzyme involved in its formation,¹⁷ represents a cardioprotective axis within the renin-angiotensin system, of which its actions balance the ACE-Ang II-Ang II type 1 receptor effects.

As a result of these findings, new therapeutic approaches targeting the ACE2-Ang-(1-7)-Mas axis have been proposed.^{18,19} For instance, it has been demonstrated that activation of endogenous ACE2 using a small molecule called XNT reverses the cardiac fibrosis observed in spontaneously hypertensive rats, leading to a significant improvement in heart function.¹⁹ The first synthetic compound able to mimic the Ang-(1-7) effects was described by Wiemer et al¹⁸ and Santos and Ferreira.²⁰ This compound, AVE 0991, improves the

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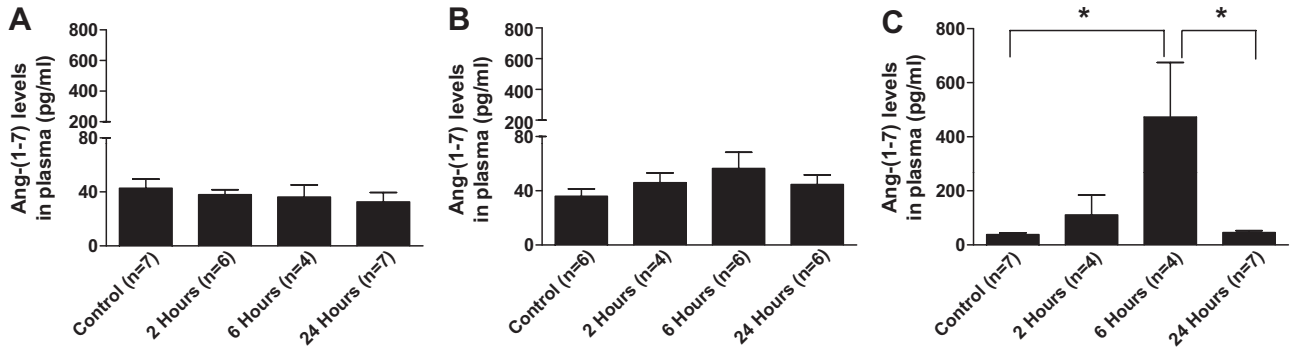


Figure 1. A, Plasma levels of Ang-(1-7) after oral administration of vehicle (control values: 42.7±6.8 pg/mL); B, Plasma levels of Ang-(1-7) after oral administration of Ang-(1-7) (control values: 35.9±5.4 pg/mL); C, Plasma level of Ang-(1-7) after oral administration of HPβCD/Ang-(1-7) (control values: 38.2±6.3 pg/mL). **P*<0.05 (1-way ANOVA followed by the Newman-Keuls posttest).

cardiac function of normal and infarcted hearts^{6,21} and protects the heart against the deleterious effects of the isoproterenol (ISO) treatment.²² Recently, it has been suggested that the inclusion of Ang-(1-7) into the oligosaccharide hydroxypropyl β-cyclodextrin (HPβCD)²³ cavity could protect the peptide during the passage through the gastrointestinal tract when orally administered. Considering the potential for using Ang-(1-7) to treat cardiovascular diseases, the aim of this study was to evaluate the cardiac effects of the HPβCD/Ang-(1-7) inclusion compound in normal, infarcted, and ISO-treated rats.

Methods

The procedures used for isolated heart perfusion, echocardiography analysis, evaluation of cardiac hypertrophy, and the area of damage induced by ISO and infarction are described in the online Data Supplement (please see at <http://hyper.ahajournals.org>).

Animals

Male Wistar rats weighing 210 to 280 g were used in this study. The animals were provided by the animal facilities of the Biological

Sciences Institute (Centro de Bioterismo, Federal University of Minas Gerais) and housed in a temperature- and humidity-controlled room maintained on a 12:12-hour light-dark schedule with free access to food and water. All of the animal procedures were performed in accordance with institutional guidelines approved by local authorities.

Effect of HPβCD/Ang-(1-7) Administration on Plasma Ang-(1-7) Levels

We conducted tests with Wistar rats to estimate the bioavailability of oral administration of aqueous formulations of free Ang-(1-7) peptide with that of the inclusion compound of Ang-(1-7) with HPβCD. The animals were euthanized and blood samples were collected before and with 2, 6, and 24 hours of vehicle (distilled water) or drug administration. Plasma Ang-(1-7) levels were evaluated using an established radioimmunoassay, according to Botelho et al.²⁴

Animal Models of Cardiac Dysfunction

The animals were submitted to 2 different maneuvers for induction of cardiac dysfunction, myocardial infarction (MI) and treatment with ISO. MI was induced by proximal left anterior descending coronary artery occlusion. In our hands, this procedure produced a

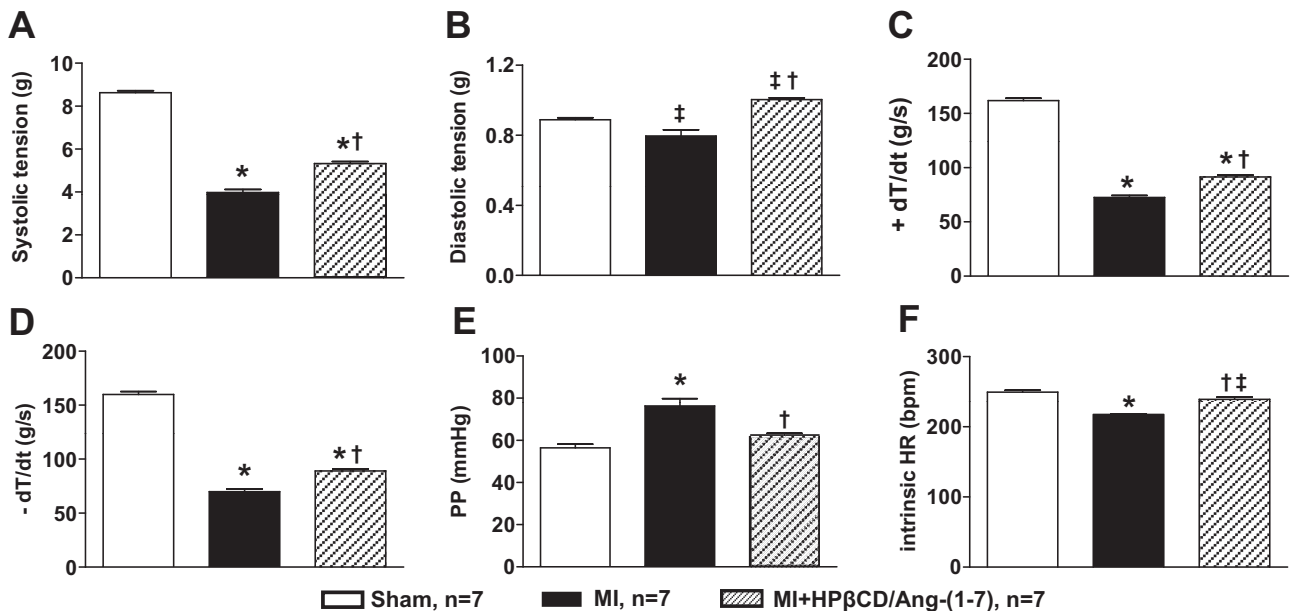


Figure 2. Effects of HPβCD/Ang-(1-7) in infarcted animals. A, Systolic tension; B, diastolic tension; C, +dT/dt; D, -dT/dt; E, coronary PP; and F, HR in isolated rat hearts perfused according to the Langendorff technique. The values of each animal were obtained by the average of 7 values (each value collected at 5-minute intervals during experimental period of 30 minutes). **P*<0.001 and ‡*P*<0.01 vs sham; †*P*<0.001 vs MI (1-way ANOVA followed by the Newman-Keuls posttest).

mild MI. The following subgroups were used in the MI protocol: sham surgery treated with HP β CD for 7 (n=7) or 50 (n=6) days; MI treated with vehicle (infarction plus HP β CD) for 7 (n=7) or 50 (n=4) days; and MI treated with Ang-(1-7) (infarction plus HP β CD/Ang-[1-7]) for 7 (n=7) or 50 (n=4) days. The groups treated for 50 days were used only for in vivo functional analysis using high-resolution echocardiography (Vevo 2100, VisualSonics, Toronto, Ontario, Canada). In the heart failure induced by ISO administration (2 mg/kg per day IP, 7 days) the following subgroups were used: control (HP β CD plus saline IP; n=8); ISO (HP β CD plus ISO; n=8); Ang-(1-7) (HP β CD/Ang-[1-7] plus saline IP; n=7); and ISO+Ang-(1-7) (HP β CD/Ang-[1-7] plus ISO; n=8). The treatment with vehicle (HP β CD; 46 μ g/kg per day in distilled water by gavage) or HP β CD/Ang-(1-7) (76 μ g/kg per day in distilled water by gavage) started on the first day of MI or ISO treatment, and the experimental procedures were performed at the end of the 7-day treatment period (isolated heart preparation) or for \leq 50 days after the beginning of the treatment (echocardiography groups). The final volumes of gavage (HP β CD and HP β CD/Ang-[1-7]) and intraperitoneal injection (saline and ISO) were \approx 0.5 mL and 0.2 mL, respectively.

Myocardial Infarction

MI was performed under anesthesia with 10% ketamine-2% xylazine (4:3, 0.1 mL/100 g, IP). The animals were placed in supine position on a surgical table, tracheotomized, intubated, and ventilated with room air using a respirator for small rodents. Subdermal electrodes were placed to allow the determination of ECG (Cardiofax, Nihon Kohden). The chest was opened by a left thoracotomy at the third or fourth intercostal space. After incision of the pericardium, the heart was quickly removed from the thoracic cavity and moved to the left to allow access to the proximal left anterior descending coronary artery. A 4-0 silk suture was snared around the left anterior descending and carefully ligated to occlude the vessel. The heart was then placed back, and the chest was closed with 4-0 silk sutures. Sham-operated rats were treated in the same manner, but the coronary artery was not ligated. After surgical procedures, ECG tracings were obtained to confirm the myocardial ischemia through ST-segment elevation and an increase in R-wave amplitude.

ISO Treatment

Heart dysfunction was induced by administration of ISO (2 mg/kg per day, IP) diluted in saline for 7 days.^{22,25} This dose of ISO was chosen because, after 7 days of treatment, it induces significant changes in cardiac histology followed by deleterious effects in heart function with low mortality rates.²⁵ To test whether the HP β CD altered the blood pressure in normotensive control rats or in the ISO-treated rats, additional conscious animals (n=5 per group) had their mean arterial pressure measured indirectly by tail-cuff method (RTBP 2000 Kent-Scientific USA) before and after the treatment. Windaq Acquisition 1.58 and Windaq Analysis 2.29 software (DATAQ Instruments DI200AC) were used to record and analyze the data, respectively.

Statistical Analysis

All of the data are expressed as mean \pm SEM. The cardiac function values of each animal were obtained through the average of 7 values (each value collected at 5-minute intervals during the 30-minute experimental period). The lesion area and total area of each animal were obtained by the average of all of the values acquired in each tissue section. Statistical significance was estimated using 1-way ANOVA followed by Newman-Keuls posttest. Student *t* test was used for the quantification of lesion area induced by ISO. In additional groups chronically treated, the echocardiography data were estimated using 2-way ANOVA followed by the Bonferroni posttest. The level of significance was set at $P < 0.05$ (GraphPad Prism 4.0).

Results

Effect of HP β CD/Ang-(1-7) Administration on Plasma Ang-(1-7) Levels

As shown in Figure 1, oral administration of HP β CD/Ang-(1-7) dramatically increased plasma Ang-(1-7) levels. The

heptapeptide increased 3-fold with 2 hours of administration and peaked at 6 hours (\approx 12-fold increase). No significant effects on plasma Ang-(1-7) levels were observed when vehicle or free peptide control was administered. Thus, the

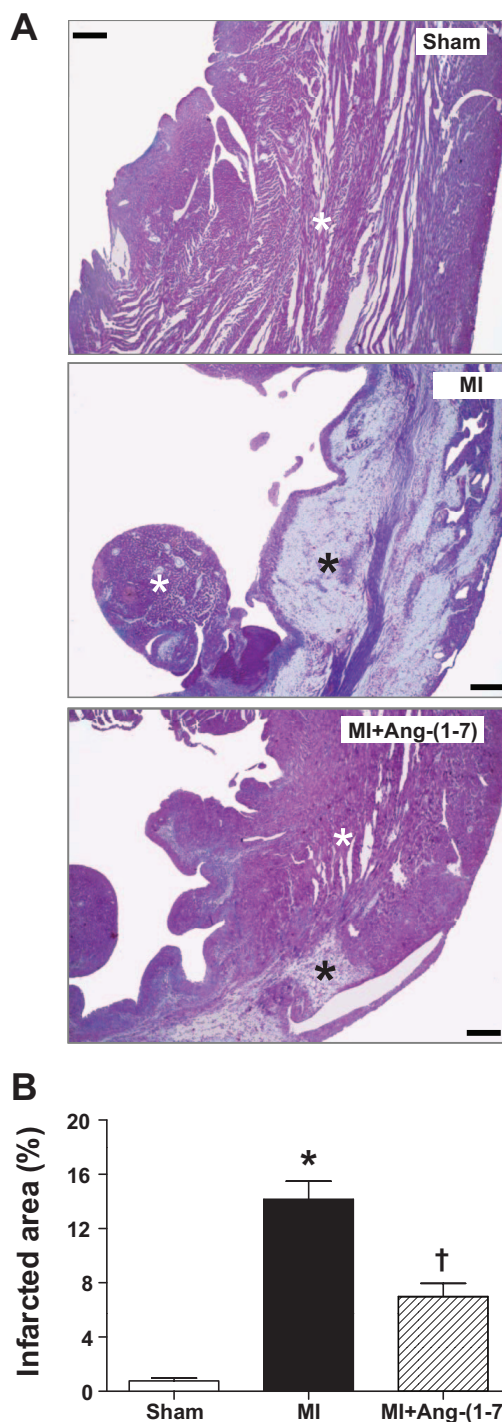


Figure 3. Infarcted area in hearts of animals treated with HP β CD associated or not with Ang-(1-7). A, Representative photomicrographs of sham, MI, and MI+Ang-(1-7) groups. White symbol (*) indicates preserved cardiac tissue, and black symbol (**) indicates fibrotic tissue. Bar=300 μ m. Stain: Masson trichrome. B, Quantification of the infarcted area. HP β CD/Ang-(1-7) treatment significantly reduced the cardiac damage induced by the left coronary artery ligation. * $P < 0.001$ vs sham; † $P < 0.001$ vs MI (1-way ANOVA followed by the Newman-Keuls posttest).

bioavailability of Ang-(1-7) delivered by HP β CD/Ang-(1-7) was dramatically increased shortly after administration and continued to increase for ≥ 6 hours after administration (Figure 1).

MI Model

No significant differences were observed in body weight and in heart and lung weights, normalized or not by the body weight, among all of the groups analyzed (please see Table S1 of the online Data Supplement at <http://hyper.ahajournals.org>). The absence of changes in the lung mass index indicated that, in our protocol, MI rats did not develop congestive heart failure. ECG tracings obtained after the surgical procedures showed an elevation in the ST segment in all of the infarcted animals, demonstrating the success of the technique used to promote MI (data not shown).

As expected, MI caused a significant decrease in the systolic and diastolic tensions, $\pm dT/dt$, and heart rate (HR) and induced an increase in perfusion pressure (PP). Treatment with HP β CD/Ang-(1-7) produced a significant improvement in all of these cardiac functional parameters in infarcted animals. Systolic tension increased 33.9% (5.33 ± 0.08 versus 3.98 ± 0.14 g in vehicle-treated rats; Figure 2A), diastolic tension 26.6% (1.00 ± 0.01 versus 0.79 ± 0.03 g in vehicle-treated rats; Figure 2B), $+dT/dt$ 26% (91.49 ± 1.78 versus 72.58 ± 1.87 g/s in vehicle-treated rats; Figure 2C), $-dT/dt$ 27.5% (89.16 ± 1.68 versus 69.95 ± 2.53 g/s in vehicle-treated rats; Figure 2D), and HR 9.9% (239.2 ± 3.2 versus 217.6 ± 0.7 bpm in vehicle-treated rats; Figure 2F). In addition, HP β CD/Ang-(1-7) completely blocked the increase in PP induced by MI (61.71 ± 1.67 versus 76.27 ± 3.55 mm Hg in vehicle-treated rats; Figure 2E).

An injured area was observed in all of the hearts of animals submitted to the MI procedure (Figure 3A). Strikingly, the

infarcted area was significantly lower in animals treated with HP β CD/Ang-(1-7) when compared with vehicle-treated rats (Figure 3A). The infarcted area quantification revealed that the HP β CD/Ang-(1-7) treatment significantly reduced the cardiac damage induced by ligation of the left coronary artery in $\approx 50\%$ (Figure 3B).

Echocardiography analysis at 7 days after MI essentially showed the same profile observed in isolated hearts experiments, that is, an improvement in the cardiac function of rats administrated with HP β CD/Ang-(1-7). Specifically, HP β CD/Ang-(1-7) increased ejection fraction and fractional shorting and decreased the systolic left ventricular (LV) volume of infarcted rats (please see Figures S1 and S2). No significant changes in stroke volume, cardiac output, and HR were observed among all of the groups (please see Figure S3). Furthermore, in infarcted animals chronically treated with HP β CD/Ang-(1-7) (50 days), the reduction of the ejection fraction and fractional shorting and the increase in the systolic and diastolic LV volumes observed in infarcted rats were significantly attenuated (please see Figures S1 and S2). Again, no significant changes were observed in the stroke volume, cardiac output, and HR among all of the groups (please see Figure S3). Of note, the progressive decrease in the ejection fraction and in the fractional shorting observed in infarcted rats was not because of changes in HR, which remained essentially constant. Thus, the progressive increase in diastolic LV volume was the main mechanism underlying the maintenance of the stroke volume in the presence of a prominent decrease in the fractional shorting.

ISO Model

ISO induced a marked decrease in cardiac function after 7 days of treatment. Animals cotreated with HP β CD/Ang-(1-7) presented an improvement in systolic tension (6.47 ± 0.05 versus

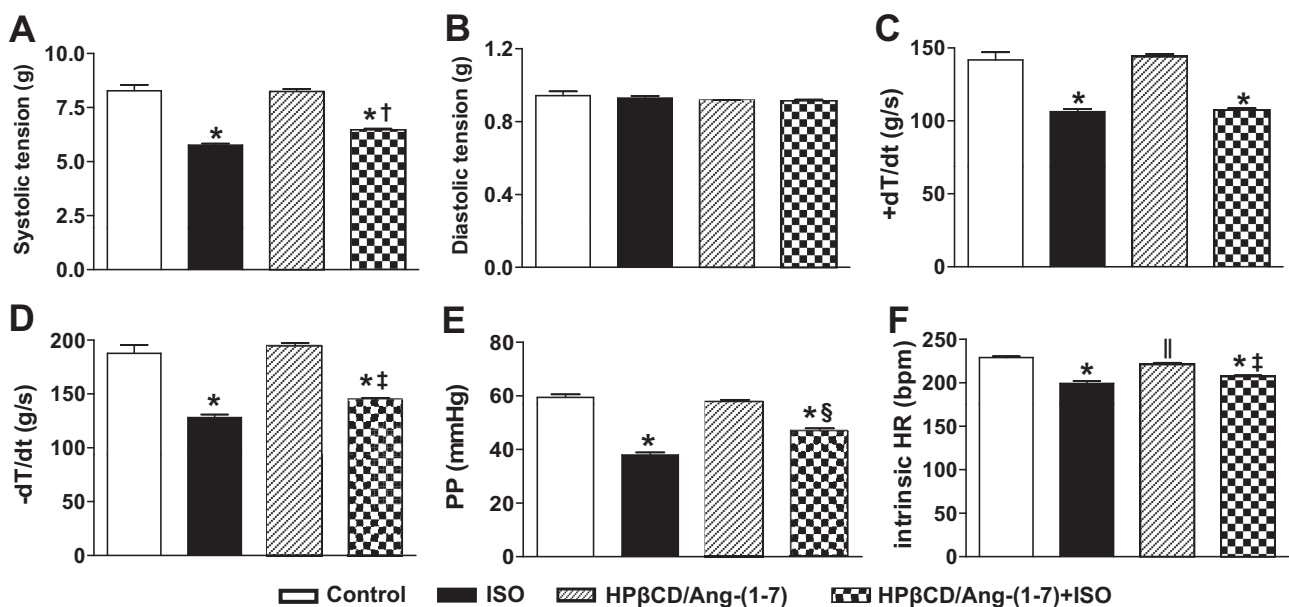


Figure 4. Effects of HP β CD/Ang-(1-7) in ISO-treated animals. A, Systolic tension; B, diastolic tension; C, $+dT/dt$; D, $-dT/dt$; E, coronary PP; and (F) HR, in isolated rat hearts perfused according to the Langendorff technique (n=8). The values of each animal were obtained by the average of 7 values (each value collected at 5-minute intervals during experimental period of 30 minutes). $\|P < 0.05$, * $P < 0.001$ vs control; $\ddagger P < 0.05$, $\dagger P < 0.01$, $\S P < 0.001$ vs ISO (1-way ANOVA followed by the Newman-Keuls posttest).

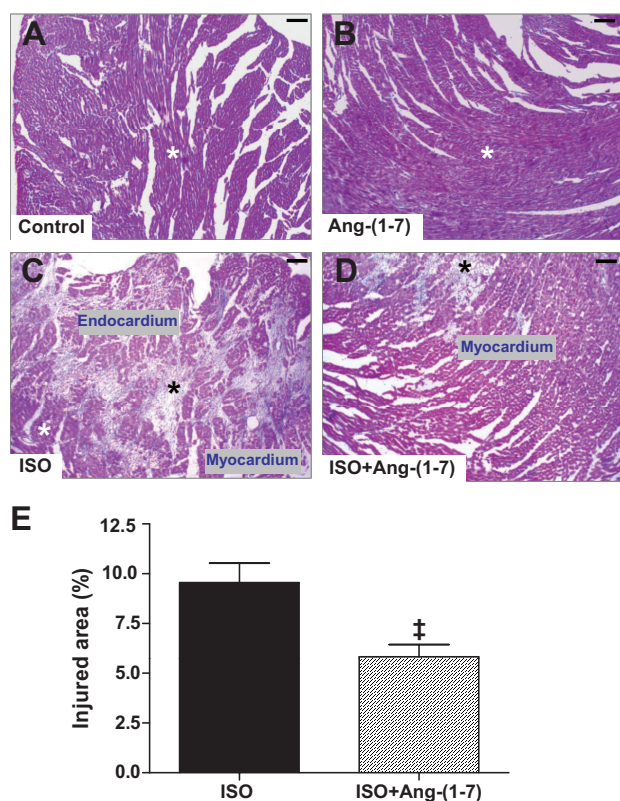


Figure 5. Effects of HP β CD/Ang-(1-7) in the damaged area induced by ISO-treatment. A–D, Representative photomicrographs of heart sections showing a significant reduction in the myocardial damaged area in animals treated with HP β CD/Ang-(1-7). White asterisk indicates preserved cardiac tissue and black asterisk indicates damaged area. Bar=100 μ m. Stain: Masson trichrome. E, Quantification of the injured area. HP β CD/Ang-(1-7) treatment significantly reduced the cardiac damage induced by ISO treatment. $\ddagger P < 0.01$ vs ISO alone (unpaired Student test).

5.76 \pm 0.07 g in vehicle-treated rats; Figure 4A), $-dT/dt$ (108 \pm 1.79 versus 96 \pm 2.06 g/s in vehicle-treated rats; Figure 4D), PP (46.8 \pm 1.10 versus 37.9 \pm 0.92 mm Hg in vehicle-treated rats; Figure 4E), and HR (207 \pm 1.55 versus 199 \pm 2.93 bpm in vehicle-treated rats; Figure 4F). However, the decrease in $+dT/dt$ caused by ISO administration was unaffected by HP β CD/Ang-(1-7) treatment (Figure 4C). No significant changes were observed in diastolic tension (Figure 4B). Hearts from rats treated only with HP β CD/Ang-(1-7) presented a slight decrease in HR.

Administration of ISO caused an extensive area of necrosis in hearts (Figure 5C), which was significantly reduced by the cotreatment with HP β CD/Ang-(1-7) (Figure 5D). As observed in Figure 5E, quantification of this effect showed a reduction of \approx 40% in the extension of the cardiac damage in the animals treated with HP β CD/Ang-(1-7). Figure 5A and 5B show heart sections from control and from an animal treated with HP β CD/Ang-(1-7) alone, respectively.

As shown in Table S2, ISO treatment induced a significant cardiac hypertrophy evidenced by the ratio between the heart weight and body weight. This effect was mainly attributed to the hypertrophy of the left ventricle because no significant differences in right ventricle/body weight and atria/body

weight ratios were observed. The ISO hypertrophic effect was confirmed by the measurement of the diameter of cardiomyocytes from free wall LV and interventricular septum (Figure 6). Administration of HP β CD/Ang-(1-7) reduced the ISO effects on the cardiomyocyte diameters by \approx 5.0% and 3.2% in free wall LV (Figure 6B) and interventricular septum (Figure 6C), respectively. However, the antihypertrophic effect of the HP β CD/Ang-(1-7) was not observed in the muscle mass index, probably because of the lower sensitivity of this method when compared with cardiomyocyte morphometry. Surprisingly, administration of HP β CD/Ang-(1-7) alone reduced the diameter of cardiomyocytes from the interventricular septum. This effect was also observed in the LV mass index, although the diameter of the cardiomyocytes of the LV wall was not altered by the treatment.

Discussion

The most important finding of the current study is that once-a-day oral administration of the inclusion compound HP β CD/Ang-(1-7) produced cardioprotective effects in 2 models of cardiac dysfunction, that is, MI and heart failure induced by ISO. This observation is in keeping with the cardioprotective role of Ang-(1-7)/Mas^{2,16} and indicated that, as suggested before, the inclusion of Ang-(1-7) in HP β CD is an effective way to orally administer this peptide.²³

It has been demonstrated that Ang-(1-7) is an important peptide involved in the regulation of blood pressure,^{26,27} cardiac function,^{6,9,21,22} cardiac remodeling,^{10,11} and cell growth,^{12,13,27,28} suggesting a potential therapeutic use of Ang-(1-7) in several disease conditions, such as cardiac hypertrophy, heart failure, hypertension, preeclampsia, and cancer. However, similar to other peptides, Ang-(1-7) could experience degradation in the gastrointestinal tract.²⁹ Indeed, our data with administration of the free peptide support this concept. To circumvent this limitation, the recent inclusion of Ang-(1-7) in HP β CD²³ allowed us to administer Ang-(1-7) orally and test its effects in rat hearts. We have observed a substantial increase in plasma Ang-(1-7) concentration 6 hours after oral administration of the inclusion compound as compared with the minor changes observed after administration of the free form of the peptide. This is in keeping with the concept that cyclodextrins can protect substances for delivery at the distal parts of the gastrointestinal tract.²⁹ The colon is a superior organ for peptide absorption after oral ingestion, and many studies indicate that colon-specific drug carriers may be used for delivering peptide drugs to that organ.²⁹ For colon-specific drug delivery, a variety of compounds, including polysaccharides, has been used. They are chemically stable, safe, nontoxic, hydrophilic, and biodegradable and, hence, excellent candidates for drug delivery systems. A large number of polysaccharides, such as cyclodextrins, has been tried for their potential as colon-specific drug carrier systems.²⁹ Here we demonstrated the feasibility of the use of HP β CD as a nanocarrier for Ang-(1-7) delivery.

Once-a-day HP β CD/Ang-(1-7) administration improved all of the functional cardiac parameters in the infarcted hearts. These findings are in accordance with previous data showing that Ang-(1-7) infusion⁹ or oral administration of its nonpep-

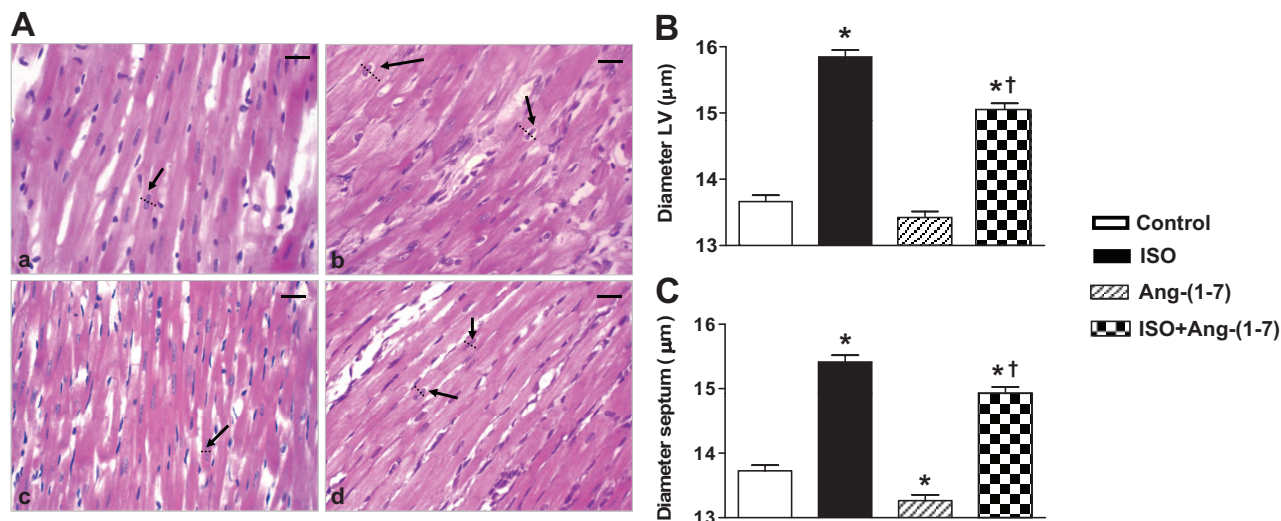


Figure 6. Effects of HP β CD/Ang-(1-7) on cardiomyocyte diameters (arrows and broken lines). A, Representative photomicrographs showing the effects of HP β CD/Ang-(1-7) on the hypertrophy induced by ISO administration. a, Control; b, ISO; c, Ang-(1-7); d, ISO+Ang-(1-7). HP β CD/Ang-(1-7) significantly reduced the hypertrophic effect of the ISO. Bar=20 μ m. Stain: hematoxylin/eosin. The increase in the cardiomyocyte diameters caused by ISO treatment in the (B) free wall LV and (C) interventricular septum was diminished by coadministration of HP β CD/Ang-(1-7). The data are shown as mean \pm SEM. * P <0.001 vs control and † P <0.001 vs ISO (1-way ANOVA followed by the Newman-Keuls posttest). LV, left ventricle.

tide mimic AVE 0991²¹ protected the heart against injuries induced by MI. For example, as observed in studies using AVE 0991,²¹ HP β CD/Ang-(1-7) administration was able to completely inhibit the vasoconstriction viewed in infarcted hearts. Even with 50 days of treatment with the inclusion compound, no evidence for toxic effects was observed. In addition, the beneficial effects of the HP β CD/Ang-(1-7) formulation were evident with long-term administration.

Cardiomyocyte diameter analysis revealed that HP β CD/Ang-(1-7) treatment reduced the hypertrophic effect induced by ISO. A similar finding was observed in transgenic animals whose plasma Ang-(1-7) levels are 2.5-fold higher²⁵ and in AVE 0991-treated rats.²² Furthermore, the treatment with the inclusion compound reduced the damaged area caused by ISO administration and, consequently, preserved the cardiac function. Many studies have shown that Ang-(1-7) possesses antitrophic and antifibrotic effects in cardiac cells.^{10–13,22,25} For instance, Grobe et al^{10,11} found that chronic infusion of Ang-(1-7) attenuates the cardiac remodeling in Ang II-treated rats¹¹ and in deoxycorticosterone acetate-salt rats.¹⁰ Of note, the antihypertrophic effect of the HP β CD/Ang-(1-7) was not observed in the muscle mass index, likely because of the lower sensitivity of this method when compared with cardiomyocyte morphometry.

In accordance with our previous study,²² ISO administration did not alter the blood pressure with 7 days of treatment, confirming that the cardiac hypertrophy observed in this model is caused by a blood pressure-independent mechanism. In addition, systemic blood pressure was not changed by HP β CD/Ang-(1-7) administration. This observation corroborates previous studies showing that the Ang-(1-7) effects on blood pressure of normotensive animals are not prominent.^{6,10,11,30}

In control hearts, HP β CD/Ang-(1-7) alone induced only a slight decrease in HR. This could be interpreted as an

unanticipated finding, because we and other have demonstrated that Ang-(1-7) and its analog AVE 0991 elicit an improvement on cardiac function of normal rats, including changes in contractility and perfusion.^{3,9,22,25} However, the possibility that the peptide, which in contrast with AVE 0991 is highly hydrosoluble, was washed out during the perfusion, probably accounts for this observation. These apparent discrepant results can also be explained, at least partially, by the final concentration of Ang-(1-7) that was achieved in the heart. Although plasma Ang-(1-7) concentration is 3-fold higher 2 hours after administration of the HP β CD/Ang-(1-7), we do not exactly know its biodistribution. Thus, future studies addressing the pharmacokinetics of this compound are essential. Unexpectedly, HP β CD/Ang-(1-7) treatment alone caused a decrease in cardiomyocyte diameters in the interventricular septum region. This finding suggests that activation of region specific antigrowth pathways could be an additional mechanism by which Ang-(1-7) induces its antihypertrophic actions.

In this study we have obtained evidence, using 2 different models, that administration of an orally active formulation of Ang-(1-7), the inclusion compound HP β CD/Ang-(1-7), produced cardioprotective effects in rats. Considering that HP β CD is essentially excreted in the feces and that the active substance entering the circulation with the use of this inclusion compound is the endogenous peptide Ang-(1-7), this may represent an important advantage of its use in comparison with synthetic derivatives. The possibility of using an orally active formulation of Ang-(1-7) opens new perspectives for the study and treatment of cardiovascular diseases.

Perspectives

The compound HP β CD/Ang-(1-7) produced beneficial effects in rat hearts, such as improvement of cardiac function (especially at 7 days of treatment), reduction of the damaged

area caused by MI and ISO treatment, and reduction of the cardiac hypertrophy induced by ISO. Thus, our current findings confirm and extend previous data demonstrating the beneficial effects of this peptide in hearts and, more importantly, indicate that this inclusion compound is a feasible formulation for oral administration of Ang-(1-7). Altogether, these results suggest that HP β CD/Ang-(1-7) could be considered as a putative new and innovative therapeutic drug for treatment of cardiovascular diseases.

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Disclosures

None.

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ONLINE SUPPLEMENT

AN ORAL FORMULATION OF ANGIOTENSIN-(1-7) PRODUCES CARDIOPROTECTIVE EFFECTS IN INFARCTED AND ISOPROTERENOL- TREATED RATS

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Short title: Oral formulation of Ang-(1-7) and cardioprotection

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Methods

Isolated Heart Perfusion

At the end of the period of treatment (7 days), the animals were decapitated 10-15min after intraperitoneal injection of 400 IU of heparin. After the thorax was opened, the heart was carefully dissected, removed from the thoracic cavity, and placed in a plate containing ice-cold Krebs-Ringer solution (KRS) to attenuate any potential cardiac damage during dissection of aorta artery. The hearts were perfused through an aortic stump with KRS containing (in mmol/L): 118.4 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄·7H₂O, 2.5 CaCl₂·2H₂O, 117.0 glucose and 26.5 NaHCO₂. The perfusion flow was maintained constant (7-8 ml/min) at 37±1°C, as well as the oxygenation (5% CO₂-95% O₂). A force transducer was attached to the apex of the ventricles through a heart clip in order to record the contractile force (tension, g) on a computer, using a data acquisition system (Biopac Systems, Santa Barbara, CA). A diastolic tension of 0.5–1.0 g was applied to the hearts. Coronary perfusion pressure (PP) was measured by means of a pressure transducer connected to the aortic cannula and coupled to the recording system. Heart rate (HR) and dT/dt were derived from the changes in cardiac tension. After 30 min of stabilization, functional parameters (both systolic and diastolic tensions, ±dT/dt, PP and HR) were recorded for an additional period of 30 min.^{1,2}

Echocardiography Analysis

In additional groups chronically treated with HPβCD/Ang-(1-7), animals underwent transthoracic echocardiographic examination under anesthesia with isoflurane before the surgery, and after 7, 15, 30 and 50 days of left coronary artery ligation performed under 10%ketamine/2%xylozine anesthesia. In vivo cardiac morphology and function were assessed noninvasively using a high-frequency, high-resolution echocardiography system consisting of a VEVO 2100 ultrasound machine equipped with 16-40 MHz transducers (Visual Sonics, Toronto, Canada). Rats were anaesthetized using 3.5% isoflurane for induction, the anterior chest was shaved and rats were placed in supine position in an imaging stage equipped with built-in electrocardiography electrodes for continuous heart rate monitoring and a heater to maintain the body temperature at 37° C. Anesthesia was sustained via nose cone with 2.5% isoflurane. High-resolution images were obtained in the right and left parasternal long and short axis and apical orientations. Standard B-mode images of the heart and pulsed Doppler images of the mitral and tricuspid inflow were acquired. Left ventricular dimensions and wall thickness were measured at the level of the papillary muscles in left and right parasternal short axis, at end-systole and end-diastole. Left ventricular ejection fraction (LVEF), fractional shorting (LVFS) and mass were measured. All the measurements and calculations were done in accordance with the American Society of Echocardiography. The following M-mode measurements were done: LV internal dimensions at both diastole and systole (LVIDd and LVIDs respectively), LV posterior wall dimensions at diastole and systole (LVPWd and LVPWs respectively), interventricular septal dimensions at both diastole and systole (IVSd and IVSs, respectively). From these measurements; end diastolic and end systolic volumes, (EDV and ESV), fractional shorting (FS), ejection fraction (EF) of the LV, stroke volume (SV), cardiac output (CO) were derived). Isovolumetric relaxation time and aortic ejection time were acquired in four chamber view and short axis view respectively. Also, it was performed using Vevostrain software, radial strain from bidimensional long axis view of left ventricle. Velocity, displacement, strain and strain rate were measured.

Cardiac Hypertrophy and Damage Induced by Isoproterenol

Heart hypertrophy was evaluated by muscle mass index (n=11 per group) and morphometry (n=7 per group). At the end of heart perfusion, the atria, right ventricles (RV) and left ventricles (LV) were dissected. The wet weights of hearts and chambers were recorded, normalized for body weight and then expressed as muscle mass index (mg/g). LV were placed in 4% Bouin's fixative for 24 hours at room temperature. The tissues were dehydrated by sequential washes with 70, 80, 90 and 100% ethanol and embedded in paraffin. Longitudinal sections (4 μ m) were cut starting from median area of the LV at intervals of 40 μ m and stained with hematoxylin-eosin for cell morphometry. Cardiac hypertrophy was evaluated with a light microscope (BX 60, Olympus) through a ruler, adapted to the eyepiece, whose each millimeter corresponds to 2.5 μ m. The cardiomyocytes were observed with a 40X magnification objective and 100 cells per animal from the septum (S) region and from the LV free wall were measured. Only cardiomyocytes whose nuclei and cellular limits were well defined were measured. Transversal sections (4 μ m) were obtained from the LV at intervals of 40 μ m and stained with Masson's trichrome to visualize the damaged tissue. For quantification of the damaged area, two sections from each animal (n=14) were used. These areas were expressed in percentage since the total area of the sections was also measured. Images with 10X and 5X magnification were captured using a JVC TK-1270/RGB micro camera to measure damaged area and total tissue area, respectively. The KS300 software in a Kontron Elektronik/Carl Zeiss image analyzer was used for damaged and total tissue area quantification utilizing the image segmentation function, whose pixels with blue hues were selected for the creation of a binary image and subsequent calculation of the corresponding areas.¹ The data were expressed in square millimeters.

Infarcted Area Analysis

After decapitation, the wet weights of the lungs were recorded, normalized for body weight and then expressed as lung mass index (mg/g) for evaluation of cardiorespiratory dysfunction. A similar procedure was performed for the hearts at the end of perfusion. Subsequently, the hearts were divided into three portions (base, medium and apex), which were submitted to the same conditions of fixation (4% Bouin), dehydration and inclusion as mentioned above. Transversal sections (4 μ m) were obtained from the medium portion, starting from the point immediately below the occlusion of the coronary artery towards the apex. These sections were set at intervals of 40 μ m. Masson's trichrome stain was used for infarcted and total tissue area quantification. Two transversal sections were evaluated per animal (n=7 per group). Image capture and analysis were made as mentioned before.

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- 2 Ferreira AJ, Oliveira TL, Castro MCM, Almeida AP, Castro HC, Caliari MV, Gava E, Kitten GT, Santos RAS. Isoproterenol-induced impairment of heart function and

remodeling are attenuated by the nonpeptide angiotensin-(1-7) analogue AVE 0991.
Life Sci. 2007;81:916-923.

SUPPLEMENTAL TABLE S1 – Morphological parameters in infarcted rats treated or not with HP β CD/Ang-(1-7).

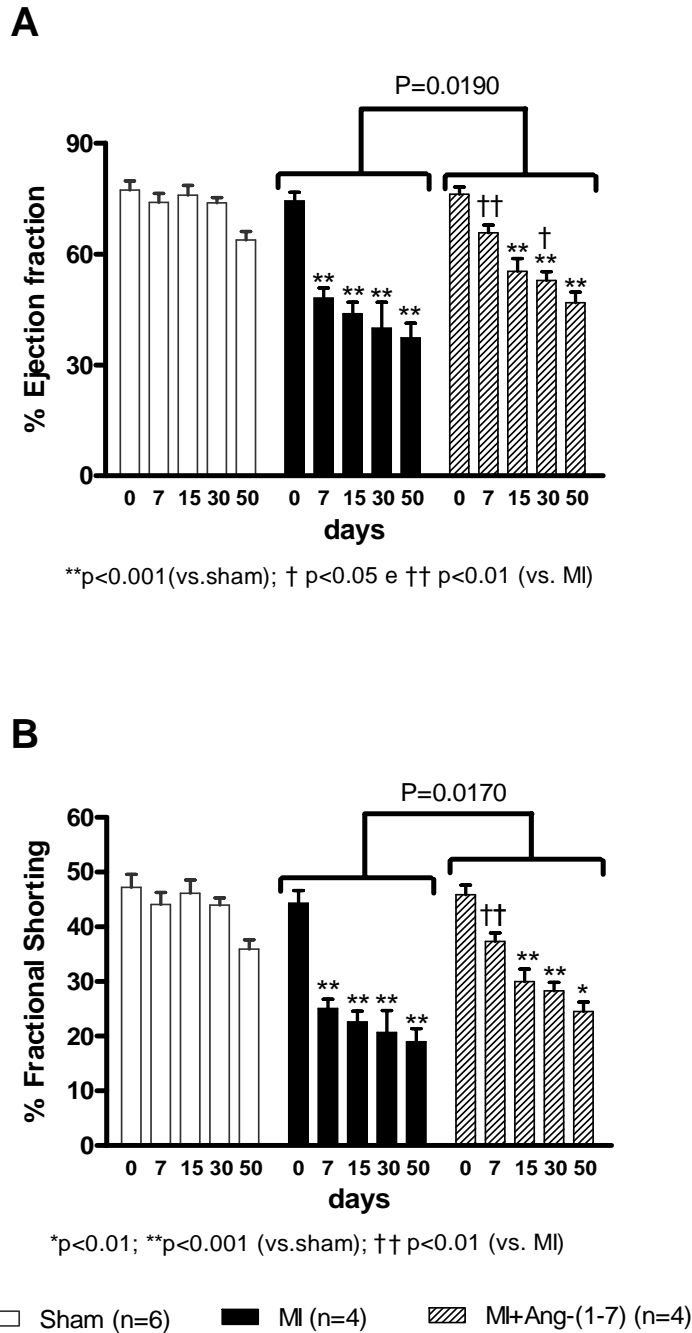
Parameters	Sham	MI	MI+Ang-(1-7)
Body weight (g)	239 \pm 5	236 \pm 15	224 \pm 16
Heart weight (g)	1.18 \pm 0.04	1.39 \pm 0.06	1.32 \pm 0.12
Lung weight (g)	1.77 \pm 0.19	1.71 \pm 0.26	1.82 \pm 0.32
Heart weight/body weight (mg/g)	4.96 \pm 0.23	5.94 \pm 0.31	6.09 \pm 0.47
Lung weight/body weight (mg/g)	7.45 \pm 0.94	7.09 \pm 0.72	8.31 \pm 1.13

Data are expressed as mean \pm SEM (n=5). No significant differences were observed among any of the groups (One-way ANOVA followed by the Newman-Keuls post test). MI, myocardial infarction.

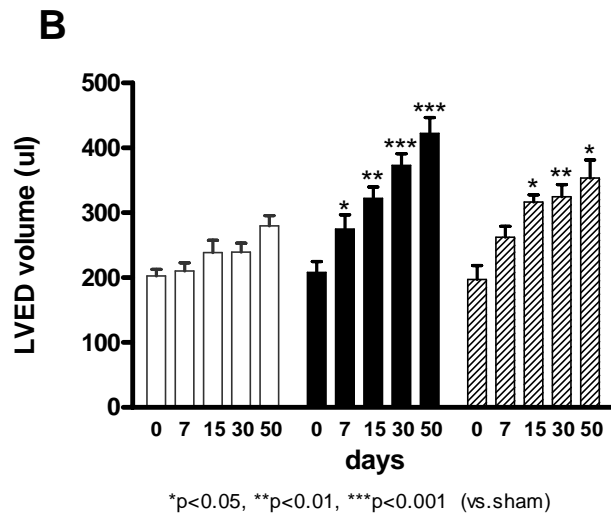
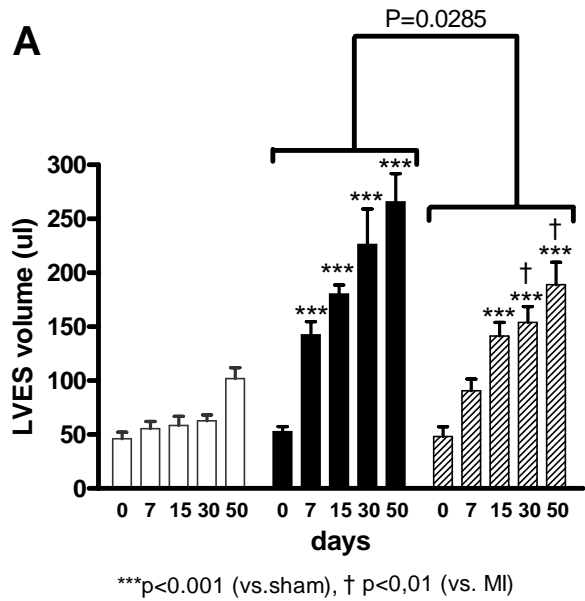
SUPPLEMENTAL TABLE S2 – Morphological parameters in rats administered with ISO, treated or not with HP β CD Ang-(1-7).

Parameters	Control	ISO	Ang-(1-7)	ISO+Ang-(1-7)
BW (g)	243 \pm 8	264 \pm 9	254 \pm 19	257 \pm 11
HW/BW (mg/g)	4.83 \pm 0.21	5.57 \pm 0.18*	4.60 \pm 0.15§	5.23 \pm 0.19
LVW/BW (mg/g)	2.66 \pm 0.07	3.15 \pm 0.08‡	2.29 \pm 0.08†	3.06 \pm 0.06‡
RVW/BW (mg/g)	0.67 \pm 0.03	0.79 \pm 0.03#	0.62 \pm 0.03	0.78 \pm 0.04#
ATW/BW (mg/g)	0.64 \pm 0.07	0.78 \pm 0.08	0.71 \pm 0.04	0.75 \pm 0.08

Data are shown as mean \pm SEM (n=11) of the body weight (BW), heart weight/body weight ratio (HW/BW), left ventricle weight/body weight ratio (LVW/BW), right ventricle weight/body weight ratio (RVW/BW) and atria weight/body weight ratio (ATW/BW). * p <0.05, † p <0.01, ‡ p <0.001 vs. Control; § p <0.01 vs. ISO, || p <0.001 vs. ISO; # p <0.001 vs. HP β CD/Ang-(1-7) (One-way ANOVA followed by the Newman-Keuls post test). ISO, isoproterenol.

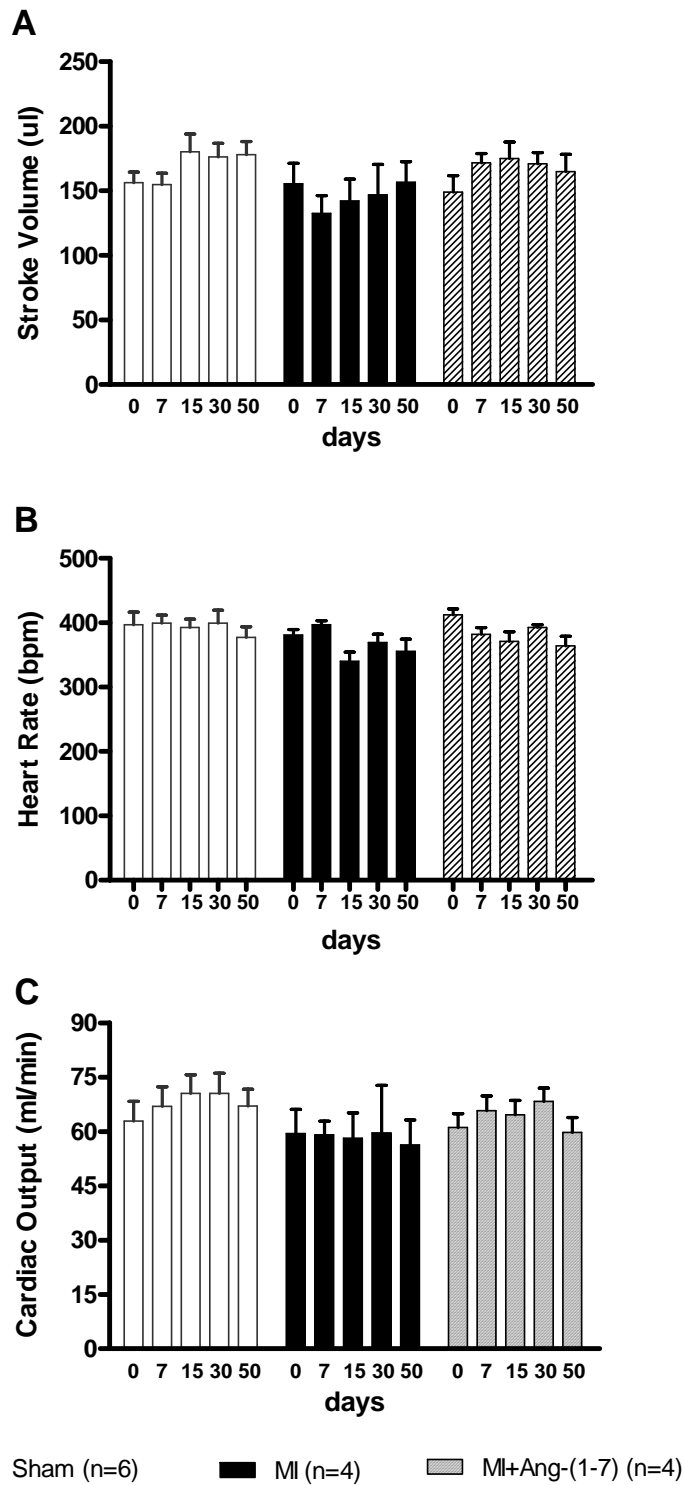


SUPPLEMENTAL FIGURE S1 - Effects of HPβCD/Ang-(1-7) on **ejection fraction (A)** and **fractional shorting (B)** after left coronary artery ligation in animals followed for up to 50 days after infarction. * p <0.01 and ** p <0.001 vs. sham; † p <0.05, †† p <0.01 vs. MI (Two-way ANOVA followed by the Bonferroni post test). The left coronary artery ligation induced a progressive decline in EF and FS of the both non-treated and **HPβCD/Ang-(1-7)-treated** groups; however the treatment reduced significantly the time-dependent decrease in these parameters.



□ Sham (n=6) ■ MI (n=4) ▨ MI+Ang-(1-7) (n=4)

SUPPLEMENTAL FIGURE S2 – Effects of HPβCD/Ang-(1-7) in **LVES (A)** and **LVED volume (B)** in animals followed for up to 50 days after infarction. * p <0.05, ** p <0.01 and *** p <0.001 vs. sham; † p <0.01 vs. MI (Two-way ANOVA followed by the Bonferroni post test). There were a progressive increase in LVED and LVES volume, which were attenuated by treatment with HPβCD/Ang-(1-7).



SUPPLEMENTAL FIGURE S3 - Effects of HPβCD/Ang-(1-7) on cardiac parameters in animals followed for up to 50 days after infarction. **(A) Stroke Volume, (B) Heart Rate, (C) Cardiac Output.** No significant difference were observed ($p>0.05$; Two-way ANOVA).