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Research Paper

Enhancement of amlodipine cardioprotection by quercetin in ischaemia/reperfusion injury in rats

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Abstract

Objectives To investigate the possible modification of the cardioprotective effect of amlodipine when co-administered with quercetin in myocardial ischaemia/reperfusion-induced functional, metabolic and cellular alterations in rats.

Methods Oral doses of amlodipine (15 mg/kg) and quercetin (5 mg/kg), alone or in combination, were administered once daily for 1 week. Rats were then subjected to myocardial ischaemia/reperfusion ($35_{min}/10_{min}$). Heart rates and ventricular arrhythmias were recorded during ischaemia/reperfusion progress. At the end of reperfusion, activities of plasma creatine kinase (CK) and cardiac myeloperoxidase were determined. In addition, cardiac contents of lactate, ATP, thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and total nitrate/nitrite (NO_x) were estimated. Finally, histological examination was performed to visualize the protective cellular effects of different pretreatments.

Key findings Combined therapy provided significant improvement in the amlodipine effect toward preserving cardiac electrophysiologic functions, ATP and GSH contents as well as reducing the elevated plasma CK, cardiac TBARS and NO_x contents.

Conclusion Quercetin could add benefits to the cardioprotective effect of amlodipine against injury induced in the heart by ischaemia/reperfusion.

Keywords amlodipine; arrhythmias; ischaemia; quercetin; reperfusion

Introduction

Obstruction of coronary arteries, the main cause of ischaemic heart disease, is the leading cause of death worldwide. Post-ischaemic reperfusion of the heart occurs spontaneously following transient coronary spasm and variant or unstable angina. It also generally accompanies clinical interventions such as percutaneous transluminal angioplasty, coronary artery bypass and heart transplantation surgery.[1] Although experimental and clinical studies have demonstrated that restoration of blood flow to severely ischaemic myocardium is a prerequisite for myocardial salvage, it was found to be associated with severe myocardial damage. This damage can be attributed mainly to formation of reactive oxygen species (ROS), intracellular calcium overload, loss of membrane integrity as well as polymorphonuclear leukocytes accumulation.[2,3] The so-called reperfusion damage includes low coronary reflow, enhanced vascular permeability, arrhythmias, myocardial stunning and acute myocardial infarction.[4]

Calcium channel blockers (CCBs) generally improve myocardial oxygenation by unloading the heart, increasing coronary blood flow and reducing myocardial oxygen consumption.[5] Interestingly, amlodipine (AMLO) has additional actions that are independent of CCB activity. AMLO was found to exhibit antiproliferative[6] and antioxidant activities[7] and the ability to stimulate nitric oxide (NO) release via endothelial nitric oxide synthase (eNOS) activation.[8]

Quercetin (QN) is one of the most abundant flavonoids found in the human diet. The association between flavonoid intake and long-term effects on mortality has been studied and it was found that flavonoid intake is inversely correlated with mortality due to coronary heart disease.[9] QN exhibits potent free radical-scavenging and metal-chelating activities.[10–12] QN was found also to inhibit xanthine oxidase activity, which has an important role in oxidative injury to tissues, especially after ischaemia/reperfusion (I/R).[13]

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The goal of the present study is to explore the possible modification of the cardioprotective effects of AMLO against myocardial I/R-induced functional, metabolic and cellular alterations, by means of its combination with QN, and to understand the possible underlying mechanism(s). Heart rates (HRs) and ventricular arrhythmias were recorded during I/R progress to assess electrophysiological changes. At the end of reperfusion, activities of plasma creatine kinase (CK) and cardiac myeloperoxidase (MPO) were determined. In addition, cardiac contents of lactate, ATP, thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and total nitrate/nitrite (NO_x) were estimated to determine metabolic changes. Finally, histological examination was performed to visualize the protective cellular effects of different pretreatments.

Material and Methods

Animals

Male albino rats weighing 180–220 g were obtained from the National Cancer Institute (Cairo, Egypt) and kept for 1 week of acclimatization at the animal facility of the Faculty of Pharmacy, Cairo University. Rats were subjected to controlled temperature (25 ± 2°C) and a constant light cycle (12 h light/dark), and allowed free access to a normal chow diet and water. The study was carried out according to the international guidelines on the Care and Use of Laboratory Animals and approved by the Ethical Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University.

Chemicals

AMLO was obtained from the Alkan Pharmaceutical Company, Egypt. QN was purchased from Sigma, USA. All other used chemicals were of analytical grade.

Methods

Rats were randomly divided into five groups of ten animals each. Groups 1 and 2 received vehicle (1% Tween 80). Groups 3, 4 and 5 received AMLO (15 mg/kg), QN (5 mg/kg) or their combination, respectively. Drugs were freshly prepared in 1% Tween 80 in distilled water and orally administered daily for 1 week before the operation. The drug concentration was adjusted so that each 100 g animal body weight received 0.25 ml of drug suspension containing the required dose. Twenty-four hours after the last dose, all rats in Groups 2, 3, 4 and 5 were subjected to 35 min of myocardial ischaemia followed by reperfusion for 10 min. Thus, rats in Group 2 served as the I/R group and rats in Group 1 were sham-operated and served as the normal group.

Myocardial ischaemia/reperfusion operation

Myocardial I/R was performed as described by Dow *et al.* and Sahna *et al.*[3,14] Rats were anaesthetized with urethane (1.4 g/kg, i.p.). The trachea was cannulated for artificial respiration using a small rodent ventilator (Bioscience, UK). Subcutaneous peripheral limb electrodes were inserted and an electrocardiogram (ECG) was continuously recorded for the entire duration of the experiment using a polygraph (Letica polygraph 4006, Spain). The animal was kept warm with a heating lamp during the operation to prevent any incidence of hypothermia. The chest was opened by a left thoracotomy, the pericardium was incised and the heart was gently exteriorized. A 6/0 polypropylene suture was quickly placed under the left coronary artery approximately 2 mm from its origin. The heart was then carefully repositioned and the animal was allowed to stabilize for 10 min. Animals that showed arrhythmias in this procedure were discarded. Both ends of the ligature were passed through a silicone tube (inside diameter 3 mm and length 8 mm) and were pulled with a small haemostatic clamp. Myocardial ischaemia for 35 min was confirmed by the presence of electrocardiographic changes (ST elevation) and the visual assessment of regional cyanosis of the ischaemic region of the left ventricle. Reperfusion was initiated by removing the clamp from the silicone tube and confirmed by a colour change in the ventricular surface from cyanosis to hyperaemia.

Heart rate, arrhythmia diagnosis and electrocardiographic analysis

HR was derived from the ECG recordings and was counted during the I/R progress. Percentages of different forms of ventricular arrhythmias, namely ventricular premature (VP), bigeminy (BG), trigeminy (TG), salvo (S), ventricular tachycardia (VT) and torsade de pointes (TdP), a specific form of ventricular tachycardia, were determined according to the Lambeth conventions[15] and the percentage of total arrhythmias (TA) was calculated. In addition, the severity of arrhythmias was quantified by a scoring system.[16] Each individual heart was evaluated by means of a four-point arrhythmia score, where VP was given a score of 1, BG/TG/S a score of 2, VT a score of 3 and TdP a score of 4. Each animal was given a score that corresponded to the most severe type of arrhythmia observed in that heart.

Biochemical measurements

At the end of reperfusion, a blood sample was collected by cardiac puncture. Plasma was separated immediately for estimation of CK activity. The heart was rapidly excised and the left ventricle was separated, washed with ice-cold saline, weighed and homogenized in ice-cold saline using a homogenizer (Heidolph Diax 900, Germany) to prepare 10% homogenate. The resultant homogenate was used for determination of the activity of MPO as well as ATP, lactate, TBARS, GSH and NO_x contents.

Plasma CK activity was assessed kinetically at 340 nm using a commercially available kit (Stanbio, USA) and results were expressed as units per litre (U/litre).

Myocardial lactate was determined according to the method of Noll that depends on oxidation of lactate by lactate dehydrogenase in the presence of NAD^+.[17] The formed NADH, which reflects the lactate concentration, was measured at 340 nm using a spectrophotometer (Thermo Electron Corporation, England). Results were expressed as micromoles per gram of wet tissue (μmol/g wet tissue).

Myocardial ATP was estimated according to the method of Lowry *et al.* which depends on the reaction of ATP with glucose in the presence of $NADP^+$ and glucose-6-phosphate dehydrogenase.[18] The increase in the fluorescence due to the formed NADPH was measured at 460 nm after excitation at 365 nm, using a spectrofluorophotometer (Bio-Tek SFM

25, Swizerland). Results were expressed as micromoles per gram of wet tissue (μmol/g wet tissue).

Myocardial lipid peroxidation products were estimated by determination of the level of TBARS that were measured as malondialdehyde[19] and expressed as nanomoles per gram of wet tissue (nmol/g wet tissue).

Myocardial GSH content was determined spectrophotometrically at 412 nm using Ellman's reagent[20] and expressed as micromoles per gram of wet tissue (μmol/g wet tissue).

Myocardial MPO activity was determined kinetically at 460 nm by measuring rate of hydrogen peroxide-dependent oxidation of o-dianisidine catalyzed by MPO[21] and expressed as units per gram of wet tissue (U/g wet tissue).

Myocardial NO_x concentration was determined spectrophotometrically at 540 nm using Griess reagent after reduction of nitrate to nitrite by vanadium trichloride[22] and expressed as micromoles per gram of wet tissue (μmol/g wet tissue).

Histological examination

At the end of reperfusion, the heart was removed as a whole and the left ventricle was separated, rinsed in ice-cold saline and preserved in well sealed vials containing 10% formalin until subsequent histological examination. The specimens were then washed, dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin wax. Serial sections 5 μm thick were obtained and stained with haematoxylin–eosin (H&E). Images were captured and processed using Adobe Photoshop (version 8.0).

Statistical analysis

All values were presented as means ± SEM. Results of HR, arrhythmia score and all biochemical parameters were analyzed using the one-way analysis of variance test (one-way ANOVA) followed by the Tukey–Kramer multiple comparison test. Results of individual arrhythmia percentages were analysed using Fisher's exact test. Statistical analysis was performed using GraphPad Instat software (version 2.04). For all the statistical tests, the level of significance was fixed at $P < 0.05$.

Results

Heart rates

Myocardial I/R caused a time-dependent increase in HRs during the entire duration of ischaemia, reaching its peak at the beginning of reperfusion. Pretreatment with any of the used drugs, alone or in combination, prevented the previously mentioned increase in HR observed in the I/R group (Table 1).

Table 1 Effect of amlodipine and quercetin on heart rate

Groups	Heart rate (beats/min)		
	Pre-ischaemic stage	End-ischaemic stage (35 min from the onset of ischaemia)	Reperfusion stage (1 min from the onset of reperfusion)
I/R	314.83 ± 9.21	348.50 ± 8.75‡	359.25 ± 7.72‡
AMLO	331.88 ± 10.25	305.25 ± 9.55$^{@}$	283.00 ± 10.97$^{@,‡}$
QN	294.75 ± 15.04	310.00 ± 11.46$^{@}$	289.75 ± 14.61$^{@}$
AMLO + QN	344.13 ± 13.81	317.25 ± 11.94	299.13 ± 12.40$^{@}$

Effect of 1 week of pretreatment with amlodipine (AMLO, 15 mg/kg per day, p.o.), quercetin (QN, 5 mg/kg per day, p.o.) alone or in combination on myocardial ischaemia/reperfusion (I/R) (35 min/10 min)-induced changes in heart rates in rats. Each value represents the mean of 8–10 experiments ± SEM. $^{‡}P < 0.05$ vs pre-ischaemic stage, $^{@}P < 0.05$ vs I/R.

Ventricular arrhythmias and arrhythmia score

Myocardial I/R produced a marked increase in all forms of ventricular arrhythmias reaching 211% (TA%). BG, VT and TdP were the prominent types of arrhythmias observed. Pretreatment with AMLO, QN or their combination afforded marked inhibitions of TA%, reaching 57% for monotherapy and 62% for combined therapy (Table 2).

Concerning the severity of arrhythmias, the I/R control group recorded a score of 3.33. Pretreatment with AMLO, QN or their combination significantly reduced the severity of arrhythmia, recording scores of 1 for monotherapy and 0.63 for combined therapy (Table 2).

Biochemical parameters

Myocardial I/R produced about a four-fold increase in plasma CK activity compared to the normal group. Pretreatment with AMLO or QN significantly decreased CK activity. Moreover, combined administration of QN with AMLO provided further significant protection compared to AMLO alone (Figure 1).

Myocardial I/R significantly increased the tissue lactate content to (8.05 ± 0.38 versus 4.71 ± 0.12 μmol/g wet tissue). On the other hand, I/R markedly decreased the myocardial ATP content (3.65 ± 0.16 versus 8.65 ± 0.19 μmol/g wet tissue). Pretreatment with AMLO, QN or their combination normalized myocardial lactate content (Figure 2). Moreover, QN alone or in combination with AMLO significantly protected against cardiac ATP depletion. On the other hand, AMLO alone failed to produce significant protection against such a decrease (Figure 2).

Myocardial I/R significantly increased the tissue TBARS (181.94 ± 6.28 versus 109.66 ± 3.11 nmol/g wet tissue). AMLO significantly decreased such an increase. Moreover, QN alone or combined with AMLO afforded marked protection that was significant compared to AMLO alone (Figure 3).

I/R injury significantly reduced the myocardial GSH content (340.23 ± 12.54 versus 521.16 ± 0.04 μmol/g wet tissue). AMLO provided significant protection against such a decrease. Moreover, QN alone or in combination with AMLO showed marked protection and normalization of GSH content (Figure 3).

Myocardial I/R significantly increased MPO activity (0.89 ± 0.02 versus 0.59 ± 0.02 U/g wet tissue). AMLO alone or in combination with QN provided almost complete protection against I/R-induced increase in myocardial MPO activity. However, QN alone did not show any significant effect toward such an increase (Figure 4).

Table 2 Effect of amlodipine and quercetin on ventricular arrhythmias

Groups	Ventricular arrhythmias (%)							Arrhythmia score
	VP	BG	TG	S	VT	TdP	TA	
I/R	22.22	55.56	22.22	11.11	66.67	33.33	211.11	3.33 ± 0.18
AMLO	28.57	14.29	0	0	14.29	0	57.14	1.00 ± 0.44$^{@}$
QN	28.57	14.29	0	0	14.29	0	57.14	1.00 ± 0.44$^{@}$
AMLO + QN	50	12.5	0	0	0$^{@}$	0	62.5	0.63 ± 0.26$^{@}$

Effect of 1 week of pretreatment with amlodipine (AMLO, 15 mg/kg per day, p.o.), quercetin (QN, 5 mg/kg per day, p.o.) alone or in combination on myocardial ischaemia/reperfusion (I/R) (35 min/10 min)-induced ventricular arrhythmias in rats. VP, ventricular premature; BG, bigeminy; TG, trigeminy; S, salvos; VT, ventricular tachycardia; TdP, torsade de pointes; TA, total arrhythmias (sum of percentages of individual arrhythmias, which may be >100% because each animal can exhibit more than one type of arrhythmia). Arrhythmia score represents the mean of 7–9 experiments ± SEM. $^{@}P < 0.05$ vs I/R.

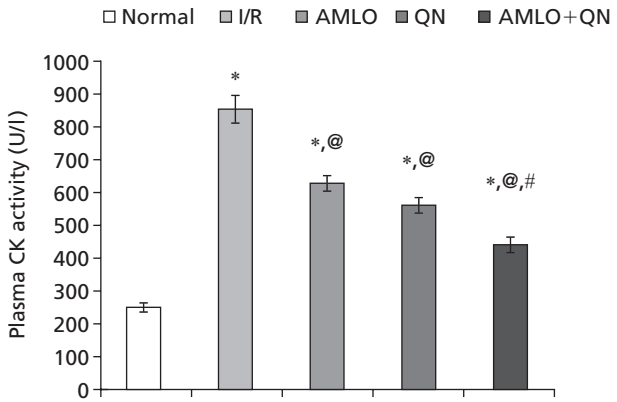

Figure 1 Effect of amlodipine and quercetin on plasma creatine kinase activity. Effect of 1 week of pretreatment with amlodipine (AMLO, 15 mg/kg per day, p.o.), quercetin (QN, 5 mg/kg per day, p.o.) alone or in combination on myocardial ischaemia/reperfusion (I/R) (35 min/10 min)-induced changes in plasma creatine kinase (CK) activity. Each value represents the mean of 7–9 experiments ± SEM. $^{*}P < 0.05$ vs control, $^{@}P < 0.05$ vs I/R, $^{\#}P < 0.05$ vs AMLO.

I/R markedly increased tissue NO_x (0.75 ± 0.02 versus 0.36 ± 0.01 μmol/g wet tissue). Pretreatment with AMLO alone showed a nonsignificant change. However, pretreatment with QN in combination with AMLO afforded complete protection against such an increase (Figure 4).

Histological examination

Myocardial I/R produced marked intercellular oedema associated with some intracellular oedema, vasodilatation and congestion as well as blood extravasation and neutrophil infiltration. In addition, waviness of some muscle fibres and apoptosis (deeply acidophilic cytoplasm with eccentric nuclei) were also observed (Figure 5). Pretreatment with AMLO afforded a degree of protection manifested by reduced intercellular oedema and cellular apoptosis (Figure 6). Pretreatment with QN reduced oedema between cardiac myocytes but neutrophil aggregation could be seen in some areas (Figure 6). Co-administration of AMLO with QN provided protection against most of the I/R-induced cardiac damage with only mild oedema observed between some cardiac myocytes (Figure 6).

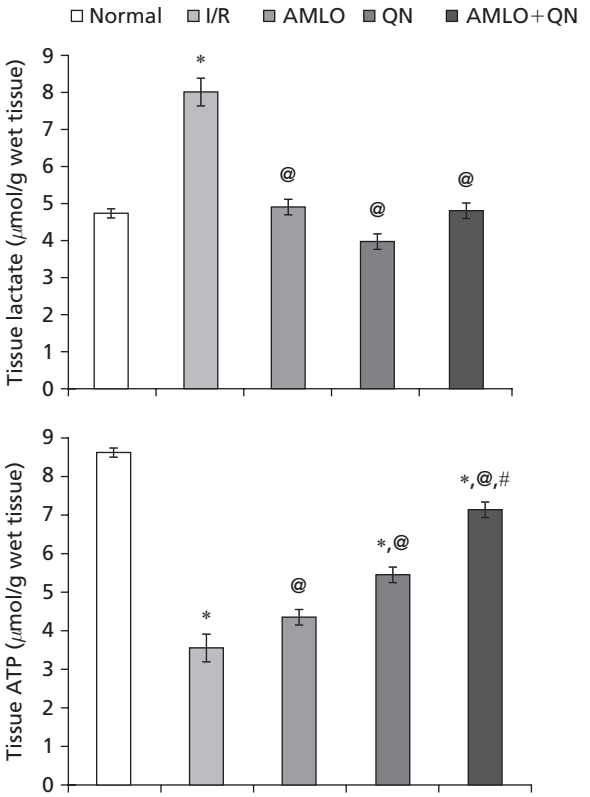

Figure 2 Effect of amlodipine and quercetin on cardiac lactate and ATP contents. Effect of 1 week of pretreatment with amlodipine (AMLO, 15 mg/kg per day, p.o.), quercetin (QN, 5 mg/kg per day, p.o.) alone or in combination on myocardial ischaemia/reperfusion (I/R) (35 min/10 min)-induced alterations in cardiac lactate and ATP contents. Each bar represents the mean of 8–10 rats ± SEM. $^{*}P < 0.05$ vs control, $^{@}P < 0.05$ vs I/R, $^{\#}P < 0.05$ vs AMLO.

Discussion

Experimental and clinical studies have demonstrated that restoration of blood flow to severely ischaemic myocardium is a prerequisite for myocardial salvage. However, restoration of blood flow to ischaemic myocardium may be associated with additional injuries to myocardium manifested at the time of reperfusion.[2] Specific biochemical, functional and ultrastructural changes are triggered by reperfusion and can

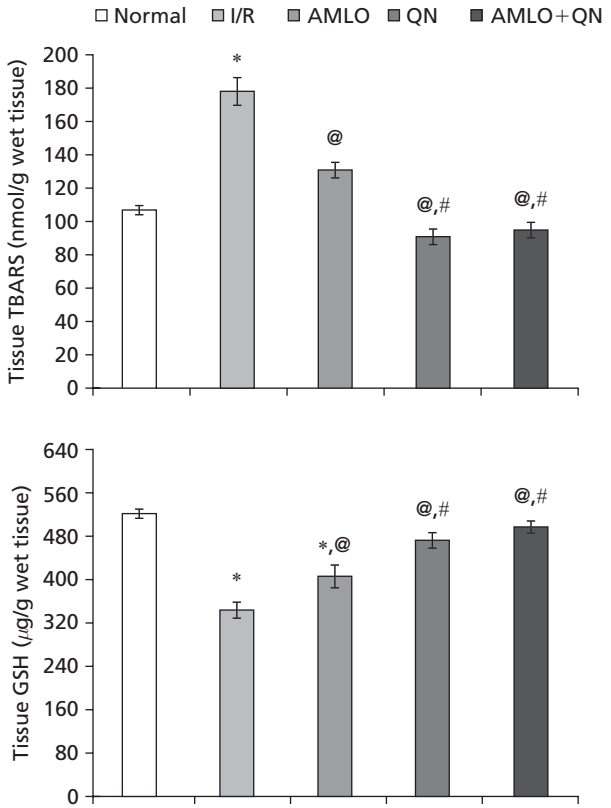

Figure 3 Effect of amlodipine and quercetin on thiobarbituric acid reactive substances and reduced glutathione contents. Effect of 1 week of pretreatment with amlodipine (AMLO, 15 mg/kg per day, p.o.), quercetin (QN, 5 mg/kg per day, p.o.) alone or in combination on myocardial ischaemia/reperfusion (I/R) (35 min/10 min)-induced alterations in cardiac thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) contents. Each bar represents the mean of 8–10 rats ± SEM. $^{*}P < 0.05$ vs control, $^{@}P < 0.05$ vs I/R, $^{\#}P < 0.05$ vs AMLO.

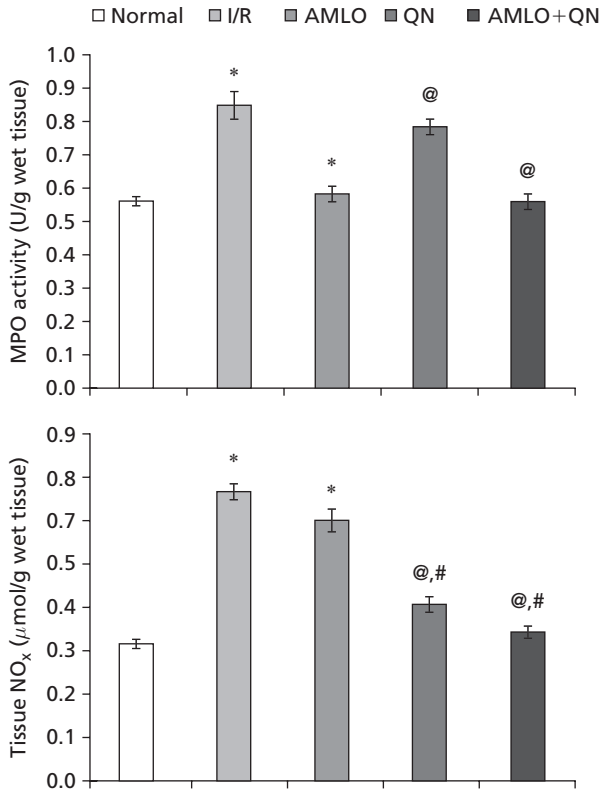

Figure 4 Effect of amlodipine and quercetin on cardiac myeloperoxidase activity and total nitrate/nitrite content. Effect of 1 week of pretreatment with amlodipine (AMLO, 15 mg/kg per day, p.o.), quercetin (QN, 5 mg/kg per day, p.o.) alone or in combination on myocardial ischaemia/reperfusion (I/R) (35 min/10 min)-induced alterations in cardiac myeloperoxidase (MPO) activity and total nitrate/nitrite (NO_x) content. Each bar represents the mean of 8–10 rats ± SEM. $^{*}P < 0.05$ vs control, $^{@}P < 0.05$ vs I/R, $^{\#}P < 0.05$ vs AMLO.

limit maximum myocardial salvage. These changes are related to calcium overload, generation of free radicals and inflammatory mediators.[4]

In the present study, the increased HR during the entire myocardial I/R can be attributed to the increase in sympathetic drive to the heart. This alteration in the autonomic balance would reduce the cardiac electrical stability, thereby increasing the incidence of ventricular arrhythmias and sudden death.[23] Prophylactic treatment with AMLO alone or in combination with QN protected against such chronotropic effects at the onset of reperfusion. CCBs have been stated to decrease HR and thereby decrease the workload on the heart.[24] The action of QN might be attributed to the membrane stabilizing properties of QN.[9]

The present myocardial I/R was associated with marked and severe ventricular arrhythmias. Similar findings have been reported previously.[25,26] These arrhythmias are supposed to be a result of heterogeneity of damage and recovery in cardiomyocytes during ischaemia and reperfusion which might lead to re-entry processes.[27] In addition, preloading the cells with positive ions, especially Ca^{2+}, may lead to the cell eliciting an ectopic contraction.[28] Moreover, local acidosis present as a result of ischaemia can lead to alterations in the cell membrane currents and promote arrhythmogenesis.[25]

Pretreatment with AMLO alone or in combination with QN provided significant protection against the incidence as well as the severity of I/R-induced ventricular arrhythmias. A number of studies have demonstrated the protective effect of CCBs against ischaemia and reperfusion-induced arrhythmias in various animal species.[29,30] Dual mechanisms (direct or indirect ones) have been suggested for attenuation of ischaemic depolarization by amlodipine. AMLO would probably reduce the degree of ischaemia and also oppose changes in intracellular ion concentration. The well-known effects of CCBs on coronary circulation and cardiac metabolism might also suggest an indirect protective mechanism.[31]

In the present study, QN provided a similar pattern of protection against I/R-induced arrhythmias. QN has been reported to act as a calmodulin antagonist, therefore it can inhibit calmodulin-dependent enzymes present at cell membrane such as ATPases and phospholipases. This would influence membrane permeability with a membrane stabilizing action.[9]

The present myocardial I/R model showed a marked elevation of plasma CK activity to about four times the preischaemic values, indicating irreversible cardiac damage

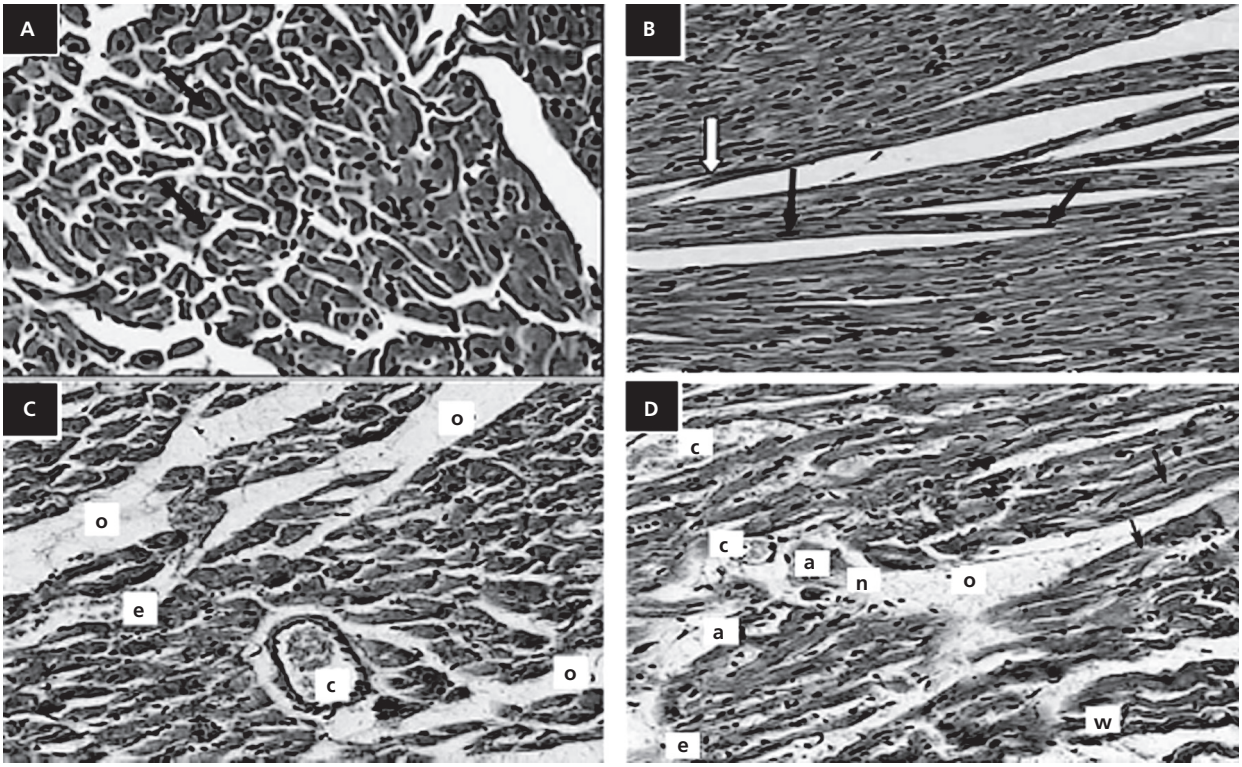

Figure 5 Histological sections of myocardial tissue stained with haematoxylin and eosin. (A) Transverse section in myocardium of normal group showing the muscle fibres with acidophilic cytoplasm and central nucleus (➡). (B) Longitudinal section in myocardium of normal group showing elongated branched acidophilic muscle fibres (⇨) with central oval nucleus (➡). (C) Transverse section in myocardium of ischaemia/reperfusion (I/R) group showing congestion (c), extravasated red blood cells (RBCs) (e) and marked oedema in between muscle fibres (o) (D) Longitudinal section in myocardium of I/R group showing congestion (c), extravasated RBCs (e), marked oedema inbetween muscle fibres (o), oedema within muscle fibre (➡), wavy muscle fibres (w) and apoptotic cells (a) and neutrophil infiltration (n). Magnification ×200.

as reported by Ishikawa *et al.*[32] This can be attributed to Ca^{2+} overload, oxidative stress and energy depletion, which would stimulate mitochondrial membrane permeability, leading to caspase activation and cellular death through apoptosis.[33] The mild improvement shown by AMLO might be attributed to attenuation of Ca^{2+} overload, one of the most important causes of cell death.[1] Moreover, the antioxidant property of AMLO might be also responsible for decreased myocardial damage and lowered plasma CK activity.[7] Concerning the protective action provided by QN, this could be related to its reported anti-apoptotic, antioxidant and metal-chelating activities.[11,12] The decreased CK activity might reflect an improved antioxidant status of animals pretreated with QN, as indicated by elevation of GSH and reduction in TBARS. This could be beneficial against oxidative stress-induced cell damage and the release of CK observed after myocardial I/R.

The present study demonstrated a significant increase in myocardial lactate together with a marked reduction in ATP content. This could be attributed to lack of oxygen and a subsequent shift from an aerobic metabolism to an anaerobic one. Moreover, ATP depletion might be attributed to mitochondrial damage with a subsequent decrease in cellular respiratory capacity and oxidative phosphorylation.[34,35] This mitochondrial damage could be exacerbated by Ca^{2+} overload and ROS generated during reperfusion.[36] In addition, ATP synthase was reported by Jennings *et al.*[37] to operate in reverse, as an ATPase during I/R, possibly contributing to a further loss of ATP during ischaemia in the present study.

The normalization of myocardial lactate that was provided by AMLO, QN or their combination could be attributed to CCB activity of AMLO as well as antioxidant properties of both. Attenuation of Ca^{2+} overload during ischaemia would decrease energy demand, thus reducing the requirement for anaerobic metabolism[1] and protecting the mitochondria from the damaging effect of ROS during reperfusion. These effects would enhance oxygen utilization and postischaemic recovery following reperfusion.[38] Moreover, this would also prevent the accumulation of NADH and thus minimize the reduction of pyruvate into lactate and prevent the incidence of acidosis.

Pretreatment with QN decreased the extent of ATP depletion. This could be attributed to its potent antioxidant and iron-chelating effects[39,40] which protect mitochondria against the damaging action of ROS during reperfusion. Furthermore, quercetin was found to alleviate the increase in mitochondrial ATPase activity following daunorubcin-induced heart mitochondrial changes.[41]

The current data revealed that combination of QN with AMLO was the most effective in alleviating the elevation of plasma CK activity and the decrease in myocardial ATP content. The reason for this approach may be due to the fact

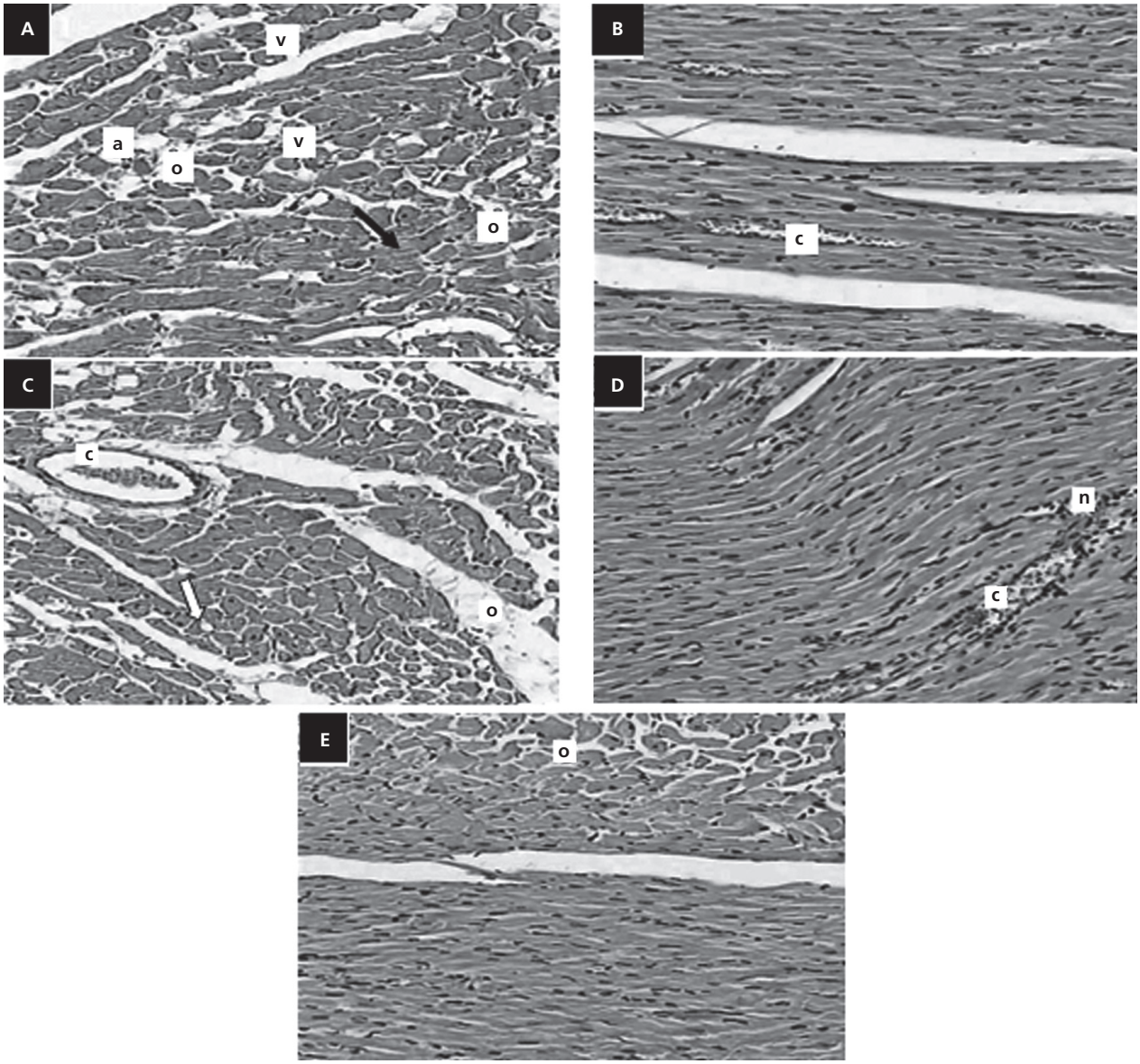

Figure 6 Histological sections of myocardial tissue pretreated with amlodipine and quercetin, stained with haematoxylin and eosin. (A) Transverse section in myocardium of amlodipine (AMLO)-treated group revealing mild oedema inbetween muscle fibre (o), mild oedema within muscle fibre (➡), vacuolated cell (v) and fewer apoptotic cells (a). (B) Longitudinal section in myocardium of AMLO-treated group showing dilated mildly congested blood vessels (c). (C) Transverse section in myocardium of quercetin (QN)-treated group showing mild dilated congested blood vessel (c), moderate oedema inbetween muscle fibres (o) and neovascularization (⇨). (D) Longitudinal section in myocardium of QN-treated group showing congested blood vessel (c) and neutrophil aggregation (n). (E) A section in myocardium of combined AMLO and QN-treated group showing transverse and longitudinal sectioned cardiac muscles fibres with mild oedema inbetween some cardiomyocytes (o). Magnification ×200.

that I/R injury is a multifactorial event in which Ca^{2+} overload is accompanied by ROS-mediated injury. Thus, effective protection was obtained by combined administration of CCB together with antioxidant.

The present study showed a state of oxidative stress following I/R. This was manifested by a significant elevation of TBARS and depletion of GSH contents. It is widely accepted that reperfusion of post-ischaemic tissues is accompanied by generation of a large amount of ROS that can overwhelm cellular antioxidant defences and induce tissue damage.[42,43] Pretreatment with AMLO afforded a degree of protection against such ROS-induced damage. The antioxidant activity of AMLO may be related to both its high lipophilicity and its chemical structure.[44] Pretreatment with QN alone or in combination with AMLO provided a marked protection against I/R-induced oxidative damage. This can be attributed to QN's potent antioxidant and iron-chelating effects, as mentioned above.

In the present investigation, myocardial I/R elevated the tissue MPO activity, indicating neutrophil accumulation as

confirmed by histological examination. Pretreatment with AMLO alone or in combination with QN normalized MPO activity. Similar findings have been reported previously by Hoshida *et al.*,[45] who treated cholesterol-fed rabbits with AMLO. Inhibition of elevated MPO activity by AMLO suggested that neutrophil infiltration was restricted. The dominant mechanism for such protection appears to be related to the ability of AMLO to donate NO. The released NO would inhibit platelet and neutrophil aggregations and hence attenuate the elevated MPO activity.[46]

In the present study, the elevation of tissue NO_x after I/R might occur mainly through overexpressed iNOS and NOS independent tissue nitrite reduction.[47] It is widely accepted that activation of neutrophils during post-ischaemic reperfusion is accompanied by release of pro-inflammatory cytokines such as TNF-α.[48] The released TNF-α within the ischaemic myocardium would downregulate eNOS and induce iNOS protein expression in the neutrophils. AMLO pretreatment showed a nonsignificant change in elevated tissue NO_x. It was previously reported that amlodipine increased cardiac NO_x following cardiac ischaemia in rats.[49] These data are directly linked to the ability of AMLO to stimulate eNOS activity.[50] AMLO protected against neutrophil aggregation in the present study, as indicated by normalized MPO activity. It therefore might be reasonable to explain the nonsignificant change of cardiac NO_x to eNOS upregulation rather than neutrophil associated iNOS overexpression. Alternatively, QN significantly decreased elevated cardiac NO_x content. This could be attributed to direct iNOS inhibition by QN, as reported by Dias *et al.*,[51] in spite of QN not attenuating leukocytic aggregation (as indicated by histological examination and elevated MPO activity). Combination of AMLO with QN almost normalized cardiac NO_x content. QN has been found to inhibit angiogenesis via suppression of overexpressed eNOS.[52] Thus, combined administration of QN with AMLO could normalize tissue NO_x that was still significantly elevated by AMLO pretreatment.

Histological examination of cardiac sections showed that combined therapy minimized the effect on cardiomyocytes, with a picture more or less similar to normal sections. This improved picture in the combined therapy was correlated with the observed alleviation in affected biochemical parameters.

Conclusions

It can be concluded that co-administration of QN with AMLO provides additional benefits to the cardioprotective potential of the latter. The cardioprotective effect of AMLO seems to be based on reduction in both oxidative stress and cell membrane damage. In addition, the cardioprotective effect of AMLO can be attributed to improvement of aerobic metabolism and attenuation of leukocytic infiltration. All of the preceding effects would lead to protection against the electrophysiological disturbances that occur during I/R. QN improved myocardial aerobic metabolism, completely counteracted the state of oxidative stress and improved both electrophysiologic cardiac function and cell viability. Combined drug pretreatment is more effective than the use of individual drugs in ameliorating the electrophysiological, biochemical and histological changes associated with I/R. Finally, clinical studies are required to establish the effectiveness of these cardioprotective agents as adjunctive therapies in patients at risk of myocardial I/R.

Declarations

Conflict of interest

The authors declare that they have no conflicts of interest to declare.

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