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Research Paper

Protective Effect of Coenzyme Q10-loaded Liposomes on the Myocardium in Rabbits with an Acute Experimental Myocardial Infarction

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Purpose. We assessed whether the infusion of Coenzyme Q10-loaded liposomes (CoQ10-L) in rabbits with an experimental myocardial infarction can result in increased intracellular delivery of CoQ10 and thus limit the fraction of the irreversibly damaged myocardium.
Methods. CoQ10-L, empty liposomes (EL), or Krebs–Henseleit (KH) buffer were administered by intracoronary infusion, followed by 30 min of occlusion and 3 h of reperfusion. Unisperse Blue dye was used to demarcate the net size of the occlusion-induced ischemic zone ("area at risk") while nitroblue tetrazolium staining was used to detect the final fraction of the irreversibly damaged myocardium within the total area at risk.
Results. The total size of the area at risk in all experimental animals was approx. 20% wt. of the left ventricle (LV). The final irreversible damage in CoQ10-L-treated animals was only ca. 30% of the total area at risk as compared with ca. 60% in the group treated with EL ($p < 0.006$) and ca. 70% in the KH buffer-treated group ($p < 0.001$).
Conclusions. CoQ10-L effectively protected the ischemic heart muscle by enhancing the intracellular delivery of CoQ10 in hypoxic cardiocytes in rabbits with an experimental myocardial infarction as evidenced by a significantly decreased fraction of the irreversibly damaged heart within the total area at risk. CoQ10-L may provide an effective exogenous source of the CoQ10 *in vivo* to protect ischemic cells

KEY WORDS: coenzyme Q10; experimental myocardial infarction; liposomes.

INTRODUCTION

Coenzyme Q10 (CoQ10), an endogenous antioxidant, is a lipophilic bezoquinone, has a key role in oxidative phosphorylation and has been reported as a potential compound for both treatment and prophylaxis of ischemia–reperfusion injury (1–3), hypertension, hyperlipidemia, coronary artery disease, and heart failure (4). CoQ10 is present in the inner mitochondrial membrane and is essential for the production of cellular energy in the form of ATP (5).

A defective myocardial energy supply due to lack of substrate and/or essential cofactors and a poor utilization efficiency of oxygen may be a common final pathway in the progression of myocardial diseases of various etiologies (6). CoQ10 serves as a highly mobile carrier of electrons between the flavoproteins and cytochromes in the mitochondrial respiratory chain (7). The fully reduced form of CoQ10, ubiquinol, inhibits lipid peroxidation in biological membranes and protects mitochondrial proteins and DNA from the oxidative damage (7,8).

It has been reported that myocardial CoQ10 and CoQ10-reductase levels are reduced in chronic cardiac failure (9), ischemia–reperfusion injury (6,10,11), cardiomyopathy (6), and other heart diseases (4,12–14). Also, levels of CoQ10 decreased during therapy with HMG-CoA reductase inhibitors, gemfibrozil, adriamycin, and certain beta-blockers (4). It has also been reported that CoQ10 reduces the depletion of ATP (15–18), diminishes the mitochondrial structural damage, (16,18,19), enhances the recovery of cardiac function after ischemia–reperfusion (16–18,20–22), decreases the incidence of reperfusion arrhythmias (23), acts a as neuroprotective against fatality (24), and protects the human low-density lipoprotein (LDL) from lipid peroxidation.

Several human trials have reported myocardial protection by CoQ10 pretreatment during coronary artery bypass surgery (9,25–28). Because of high lipophilicity and poor solubility, cellular delivery of CoQ10 is challenging. Although there are several reports published on oral delivery of CoQ10, (29,30) an immediate cellular delivery of CoQ10 would be likely to prove useful for operative cardiac interventions and for preventing the ischemia–reperfusion damage.

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ABBREVIATIONS: Ch, Cholesterol; CoQ10-L, Coenzyme Q10 liposomes; DD, Detergent dialysis; DOTAP, 1,2-dioleoyl-3-trimethyl-ammonium-propane; ECG, Electrocardiogram; ED, Ethanol dissolution; EL, Empty liposomes; EPR, Enhanced permeability and retention; KH, Kreb sHenseleit; LDL, Low-density lipoprotein; LFH, Lipid film hydration; LV, Left ventricle; NBT, Nitro blue tetrazolium; PC, Egg phosphatidylcholine; PEG-PE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; REV, Reverse phase evaporation; USB, Unispearse blue dye.

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Liposomes are widely used as nano-sized drug delivery vehicles for active and passive targeting (31). Accumulation of these lipid vesicles in regions of experimental myocardial infarction has been demonstrated (32–34), which proceeds via the enhanced permeability and retention (EPR) effect (35,36). Liposomes, especially those decorated with monoclonal antibodies specifically recognizing ischemically damaged cells, may also plug and seal the damaged myocyte membranes and protect cells against ischemic and reperfusion injury (37).

Earlier, we hypothesized that the supply of the exogenous ATP loaded into liposomes may protect the ischemic myocardium by delivering ATP to and into hypoxically compromized cells thus reducing the fraction of the irreversibly damaged heart within the total area at risk. We demonstrated such protection in an isolated rat heart model (38). This protection was associated with the accumulation of liposomes and increase in ATP level in the infarcted tissues. Our laboratory also showed that intracoronary delivery of the liposomal ATP effectively protected the myocardium from ischemia–reperfusion injury in rabbits with experimental myocardial infarction (39). An anti-ischemic action of ATP-loaded liposomes (40–42) and CoQ10-liposomes (43–46) in different tissues was also demonstrated in several other studies. However, whether acute intracoronary infusion of CoQ10 liposomes can reduce the experimental myocardial infarct size by bringing CoQ10 to and into hypoxic cardiocytes in an *in vivo* model is not clear and needs to be evaluated.

Here, we present the *in vivo* data that show a significant cardio-protective effect of CoQ10-loaded liposomes in rabbits subjected to a 30 min coronary artery occlusion. These results represent the next step in the development of methods for protection of the ischemic myocardium against the damage resulting from an inadequate energy supply.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC), cholesterol (Ch), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (PEG_{2000}-DSPE), and 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL). Coenzyme Q10, octylglycoside, and Sephadex G25 were purchased from Sigma (St. Louis, MO). All other chemicals and buffer components were of analytical grade.

Formulation Optimization of CoQ10-loaded Liposomes

In order to prepare the liposomal CoQ10 with the maximal load and stability, we have compared several different methods of liposome preparation and drug loading. CoQ10 liposomes were prepared using four different methods—lipid film hydration (LFH), reverse phase evaporation (REV), ethanol dissolution (ED), and detergent dialysis (DD). For formulation optimization, the major components of the lipid mixture were PC, Ch, and PEG-DSPE in a molar ratio of 88.5:10.5:0.5. During liposome preparation, the molar CoQ10:total lipid ratio was approximately 1:22. For these experiments, the buffer used was KH buffer without $CaCl_2$, pH 7.4. For sizing, all final liposome suspension were extruded (five times for each pore size) through two stacks of 400, and 200 nm pore size polycarbonate Nucleopore membranes using an Avanti mini-hand extruder. The encapsulated liposomal CoQ10 was always separated from the non-encapsulated free CoQ10 by gel filtration. The size and size distribution of the liposomal preparations was determined using a Coulter N4 Plus Submicron particle analyzer.

Lipid Film Hydration. Thin lipid film-hydrated vesicles were prepared according to the method described by (47). Briefly, a chloroform solution of PC, Ch, $mPEG_{2000}$-DSPE (in the above molar ratio), and CoQ10 was evaporated on a rotary vacuum evaporator, and the thin lipid film formed was hydrated with 0.5 mL of KH buffer (pH adjusted to 7.4 with 1 M NaOH) at 40°C with periodic vortexing. The liposomes were extruded five times through each of 400, and 200 nm pore size polycarbonate membranes (Nucleopore) to produce samples with a narrow size distribution.

Reverse Phase Evaporation. REV liposomes were prepared according to the method of Szoka and Papahadjopoulos (48) with some modifications. A lipid film prepared as above was dissolved in 3 mL of diethyl ether. KH buffer, 0.5 mL pH 7.4, was then added to the organic solution. The mixture was emulsified with an ultrasonicator 60 Sonic Dismembrator (Fisher Scientific) during 1 min at 10 watts. The organic solvent was then removed by incubation in a 30°C water bath for 60 min to form liposomes. Traces of diethyl ether were eliminated under vacuum. After sizing the volume was adjusted with KH buffer.

Ethanol Dissolution. All lipid-soluble components were evaporated to a thin film as explained in the lipid film hydration method. The film was then dissolved in 0.5 mL of ethanol. This mixture was vortexed for 5 min followed by sonication until a clear transparent solution was obtained. The KH buffer was added rapidly to this mixture with syringe attached with 21G needle during constant vortexing. The mixture was vortexed for an additional 5 min and extruded through polycarbonate Nucleopore membranes of 400 and 200 nm pore size with the help of an Avanti mini-hand extruder. The ethanol was removed by overnight dialysis against the KH buffer at 4°C.

Detergent Dialysis. Lipids and CoQ10 were evaporated to a thin film as explained earlier. The film was then hydrated with KH buffer containing 20 mg/mL of octylglycoside. This mixture was vortexed for 5 min. The octylglycoside was removed by dialysis against the 4 L of KH buffer at 4°C for 24 h with several buffer changes. The liposomes thus formed were sized by extruding through polycarbonate Nucleopore membranes of 400 and 200 nm pore size with the help of an Avanti mini-hand extruder.

Preparation of Liposomes for the In Vivo Rabbit Experiments. Since LFH method showed the maximum encapsulation efficiency (Table I), this method was used to prepare Coenzyme Q10 liposomes for *in vivo* rabbit experiments. Briefly, a chloroform solution of PC, Ch, and $mPEG_{2000}$-DSPE (in ratios as above), and with the addition of DOTAP (3% mol of total lipids), and CoQ10 (25% mol of total lipids) was evaporated on a rotary vacuum evaporator, and the thin lipid film formed was hydrated with 5 mL of the KH buffer without $CaCl_2$ (pH adjusted to 7.4 with 1 M NaOH) at 40°C for 60 min with periodic vortexing. The liposomes were extruded five times

Table I. Encapsulation of CoQ10 into Liposomes Using Various Methods

Method	Q10 Encapsulation Efficiency, mol%
Lipid film hydration	12.0
Reverse phase evaporation	2.8
Ethanol dissolution	7.7
Detergent dialysis	5.3

through each of 400, and 200 nm pore size polycarbonate membranes (Nucleopore) to produce samples with a narrow size distribution. These formulations were additionally supplemented with 1.7 mM $CaCl_2$ prior to infusion into the rabbit heart. The liposomal formulations (CoQ10-L) were diluted to form a preparation containing 50 mg of lipid and 12 mg of CoQ10 per mL. The size and size distribution of the liposomal preparations was determined using a Coulter N4 Plus Submicron particle analyzer.

Characterization of Liposomes. The size and size distribution of empty liposomes (EL) and CoQ10-L was determined by the dynamic light scattering using a Coulter N4 Plus Submicron Particle Analyzer (Beckman-Coulter).

Experimental Myocardial Infarction in Rabbits. The procedure was previously described elsewhere (39). Briefly, 2.5 to 3.5 kg New Zealand White rabbits (Millbrook Breeding Labs) were anesthetized s.c. with 80 mg/kg of ketamine and 8 mg/kg of xylazine, intubated via a tracheostomy, and ventilated with room air at a tidal volume of 18–22 mL at 46 to 50 strokes per minute using a Harvard rodent positive-pressure ventilator (Harvard Apparatus Co.). Anesthesia was maintained with a dilute pentobarbital Na infusion via a marginal ear vein at 20–25 mg/h. The heart was exposed through a parasternal thoracotomy. The pericardium was dissected and a flexible plastic catheter was inserted into the left atrium for rapid infusions into the coronary circulation. An anterior branch of the left coronary artery was isolated with a 3–0 suture for control of flow with an occlusive snare. The ECG was continuously monitored with a PageWriter M1700A electrocardiograph (Hewlett-Packard). Approx. 3 mL of a test suspension of CoQ10-L (a total of 133 mg of lipid and 36 mg of CoQ10), and KH buffer, pH 7.4, was infused through the coronary arteries during a brief (5–10 s) period ending in clamping of the aorta during the infusion with trapping of infusate in the area occluded by tightening the snare as the infusion ended. After 30 min, the snare was released and reperfusion established for 3 h. The coronary artery was re-occluded, and 3 mL of a 1:5 diluted Unisperse Blue (USB; Ciba) was infused via the atrial catheter to demarcate the area of the occlusion-induced ischemic zone (USB stained the normoxic perfused myocardium, while the ischemic zone termed “the area at risk” remained unstained). The anesthetized animal was immediately sacrificed by exsanguination and the heart was rapidly removed. The excised left ventricle (LV) was sliced transversely between apex and base into five to six approximately equal thickness slices, which were digitally photographed from both sides to register the site of the occlusion-induced area at risk by the absence of blue staining. Slices were then incubated for 20 min in a 40–42°C 0.05% PBS-buffered nitroblue tetrazolium (NBT) solution for the detection of the infarcted portion of the area at risk by delineation of non-infarcted areas as the stained zones containing intact myocyte dehydrogenases, while irreversibly damaged necrotic tissues within the net ischemic zone remained unstained. This technique relies on the ability of dehydrogenase enzymes and cofactors in the tissue to react with tetrazolium salts to form a formazan pigment. The slices were re-photographed and finally weighed. The area at risk and the fraction of the irreversibly damaged myocardium were determined from planimetry of both sides of all slices using Adobe Photoshop 7.0. The total LV weight at risk and the weight of the infracted LV were calculated in two independent runs and averaged. The infarction size was expressed as the percent of the total LV weight at risk.

Ethics. Protocols for this study were approved by the IACUC, Northeastern University, Boston, MA, USA, and conform to the guidelines specified in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1985).

Statistical Data Analysis. Data analysis was carried out with the software package Microcal Origin, Version 6. Results were expressed as mean±standard error, $n = 4–7$ independent samples. Statistically significant differences were determined using the analysis of variance (ANOVA) followed by Student’s t test. A p value < 0.05 was considered significant.

RESULTS

Liposome Preparation Methods and CoQ10 Encapsulation Efficiency. Since different methods of liposome preparation often yield different level of drug encapsulation, see for example (49), four different methods to prepare CoQ10-L have been tested to choose the optimal protocol. CoQ10 encapsulation efficiency into liposomes of the same composition but prepared using different methods is shown in Table I (as mol% of the incorporated CoQ10). It was observed that encapsulation of CoQ10 was significantly influenced by the method of preparation. The use of the REV method resulted in a rather lower CoQ10 encapsulation efficiency (ca. 0.028 μmol of CoQ10 per μmol of total lipid, or 2.8 mol%). The LFH method resulted in a significantly higher (ca. 4-fold higher compared to REV) CoQ10 encapsulation (ca. 0.12 μmol of CoQ10 per μmol of total lipid, or 12 mol%). ED method had an encapsulation efficiency of 7.7 mol%, and DD method resulted in the encapsulation efficiency of 5.3 mol%. Therefore, the LFH method, an efficient rather simple and inexpensive procedure, was chosen to produce CoQ10-L for further experiments.

Liposome Size and Size Distribution. The LFH method resulted in vesicles with a diameter of 167.3 ± 45.8 nm (mean ± SD) for EL and 177.2 ± 25.6 nm CoQ10-L (Fig. 1a,b). The zeta-potential value of similar lipids composition were 12.91 ± 0.41 mV (38). A slightly positive charge of the liposomal membrane may also enhance liposome interaction with cell membranes, particularly in the area of ischemic myocardium as was noted earlier (32,33).

Infarct Size in Rabbits with Experimental Myocardial Infarction. The 30 min occlusion of a branch of the left

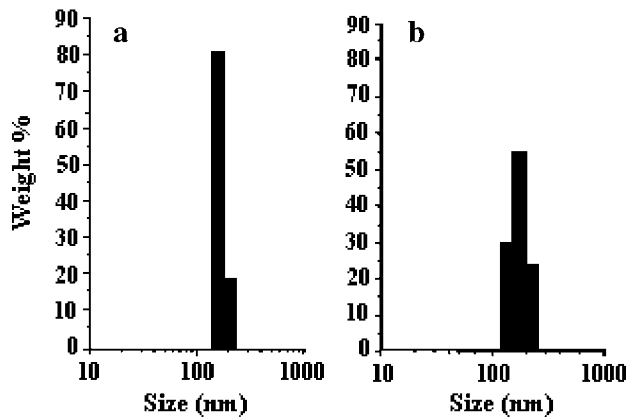

Fig. 1. The size and size distribution of EL (**a**) and CoQ10-L (**b**).

circumflex artery in rabbits resulted in myocardial ischemia as confirmed by the comparison of the S–T segments of electrocardiograms (ECG) of post-occlusion animals with pre-occlusion ECG patterns. The elevation of the S–T segment in animals with the occluded artery (39) confirmed the development of an infarction.

The average LV weight for all animals was 3.57 ± 0.11 g ($n=16$). The mean size of the area at risk (the net hypoxic area developed as a result of occlusion) for all animals was 20.4 ± 2.3% of the LV ($n=16$) as shown by the USB staining (Fig. 2A,C,E).

The effect of the CoQ10-L and control (EL, KH buffer) treatments on the size of the irreversible damage (infarction) was estimated by measuring the fraction of the area at risk (USB staining data), which by the end of the experiment underwent irreversible damage according to the NBT staining result. In control animals receiving either the EL or KH buffer, the final size of the infarction (irreversibly damaged tissue) was 59.5 ± 7.2 and 68.6 ± 3.9% of the total area at risk, respectively (Fig. 2). This 60-to-70% infarction of the area at risk after 30 min of ischemia is in good agreement with previous observations (37). At the same time, the administration of CoQ10-L resulted in a substantial decrease in the size of the irreversibly damaged myocardium within the total area at risk. The intracoronary administration of the CoQ10-L effectively limited the irreversible tissue damage to only 30.8 ± 0.9% of the total area at risk ($p=0.006$ and $p<0.001$ when compared with EL and control, respectively). Thus, in KH buffer- and EL-treated animals, the infarcted area appears almost similar to the total area at risk (see the typical data in Fig. 2, red areas in A and B; C and D), while in CoQ10-L-treated animal the developed infarct constituted a significantly smaller fraction of the total area at risk (see the typical data in Fig. 2, red areas in E and F) because of the protective action of CoQ10-L.

We have also performed an analysis of covariance for the group effect on the weight of the tissue that developed necrosis and the weight of the total area at risk. Fig. 3 shows the relationship between the absolute weight of the final necrosis and the weight of the ischemic zone. Since there was no significant difference between the final infarct size in EL- and KH buffer-treated animals (Fig. 3), these two control groups were combined for the comparison with the CoQ10-L group. There was good linear correlation between the weight of infarcted tissue and the weight of the total area at risk for the combined controls ($R^2=0.99$; $Y=-0.07+0.69X$), as well as the data for the CoQ10-L-treated group which had a distinctly lower slope ($R^2=0.95$; $Y=0.01+0.29X$; Fig. 4). The results of this analysis clearly show that the treatment with CoQ10-L significantly decreased the size of the irreversibly damaged heart tissue within the total area at risk by 50 to 55% over a weight range of the LV at risk varying from between 0.4 and 1.6 g compared to the combined controls (Fig. 4).

DISCUSSION

The results clearly demonstrate a pronounced protective effect of intracoronary infusion of CoQ10-L on the survival of the myocardium during a period of experimental acute myocardial ischemia *in vivo* resulting in an approx. 2-fold decrease in the mass of the irreversibly damaged area of the heart (revealed by the NBT staining) within the total size of the area at risk (revealed by the USB staining), when compared to the animals receiving control treatments with KH buffer or EL (Figs. 3 and 4). The 50% reduction of

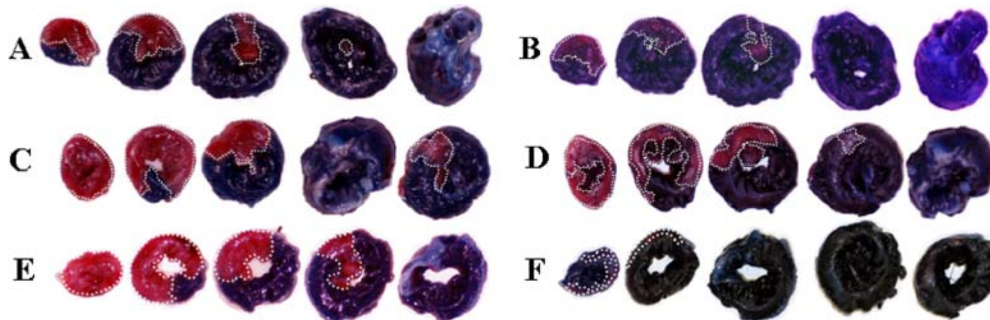

Fig. 2. USB- and NTB-stained sections of infarcted myocardium. Cardioprotective effect of CoQ10-L after 30 min of coronary occlusion and following 3 h of reperfusion in rabbits with an acute experimental myocardial infarction. *A* and *B*—Control KH buffer treated animal; *C* and *D*—EL treated animal, *E* and *F*—CoQ10-L treated animal. A, *C*, and *E*—area at risk (USB-unstained red tissue) developed as a result of occlusion; *B*, *D*, and *F*—infarcted area at the end of occlusion/reperfusion experiment (NBT-unstained tissue).

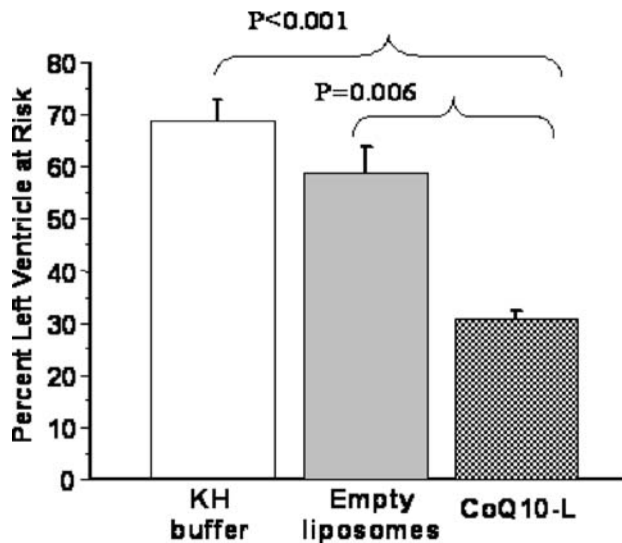

Fig. 3. Summary graph showing the fraction of the infarcted ventricle as a percentage of the total area at risk in the KH buffer-treated control group, EL and CoQ10-L-treated group. (mean±SE), $n = 4$–7.

infarct size represents the recovery of a significantly larger portion of the ventricular myocardium (ca. 10% of left myocardial mass) which could in turn be sufficient to effect a significantly enhanced potential for recovery from an infarction in a clinical situation.

It is known that the most effective approach for decreasing the degree of myocardial necrosis is the quick and complete coronary artery reperfusion. Further attenuation of myocardial necrosis can be achieved if timely reperfusion is supplemented with other interventions, such as an augmentation of the collateral blood flow, slowing metabolic degradation of ATP, reducing reperfusion injury (50), or the supply of the exogenous CoQ10 using, for example, a delivery system like liposomes that protects its payload from degradation by enzymes (38). The administration of CoQ10 with a liposomal delivery system clearly protected the ischemic heart after 30 min of coronary occlusion followed by 3 h of reperfusion.

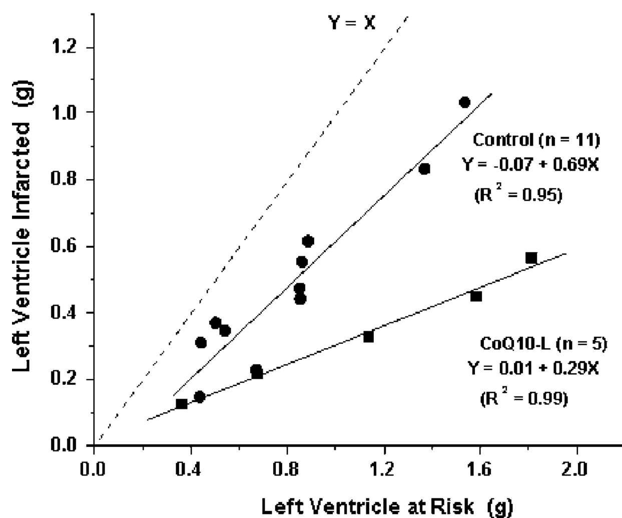

Fig. 4. Scatter plot showing the relationship between the weight of ischemic myocardium at risk and necrotic zone of the combined controls and CoQ10-L treated groups.

The coenzyme Q10 dose, used in our experiment (a total intracoronary administered dose of 36 mg CoQ10 in these experiments), was comparable to the dose used in other studies that reported beneficial effects of acute administration of CoQ10 (5–15 mg/kg), (9,18–20,22,23). It was reported earlier that the CoQ10 content of the myocardium increased in a dose-dependent manner at 60 min after the intravenous infusion of 5 or 15 mg/kg CoQ10 (23). Moreover, an earlier reported study revealed that intravenous administration of 10 mg/kg of CoQ10, given to rats 10 min before excising the hearts was sufficient to improve the recovery of the heart function during the reperfusion in an isolated heart model (22). Thus, in our experiments, the 30 min interval between administration of CoQ10 and reperfusion was probably enough to allow significant liposomal CoQ10 to enter the cardiac myocytes.

Several published reports have shown that CoQ10 pretreatment protects myocardium from an infarct/reperfusion injury via both antioxidant and bioenergetic pathways (51). It has also been shown that CoQ10 protected against oxidative inactivation of creatine kinase during reperfusion (52), and reduced creatine kinase leak from the ischemic myocardium (9,26,53,54). Elevated levels of ATP and phosphocreatine, and improved myocardial aerobic efficiency during reperfusion were observed with CoQ10 pretreatment when followed by the ischemic–reperfusion insult (52). From these reports and our current data supported by our previous experimental data from the isolated rat heart model (38), and from the rabbits with experimental myocardium infarction (39) we suggest that the infusion of CoQ10-L eventually resulted in an elevated level of CoQ10 in cardiomyocytes under the conditions of the coronary occlusion in this rabbit myocardial infarction model. Thirty minutes of occlusion may have provided a sufficient time for CoQ10-L to accumulate in the ischemic zone via the reported EPR effect (34,36), and "unload" at least a part of the liposomal CoQ10 into the ischemic cells in concentrations sufficient to protect them from irreversible damage. Our data is consistent with other published reports showing attenuation of the post-ischemia dysfunction (18,20) and the morphologic features of injury (16,18,19) by CoQ10 following the ischemic–reperfusion insult.

The mechanism of the protective action of CoQ10-L on the ischemic myocardium may also involve the combination of a non-specific plugging and sealing of the damaged cell membranes (37) with the delivery of the CoQ10 into the ischemic myocytes at the same time (38). Earlier reported results support our hypothesis on the intracellular delivery of CoQ10 by CoQ10-L (38,39).

Liposomes have been shown to cross the capillary endothelium and deliver their payload intracellularly by passing through the endothelial tight junction and by increased endothelial endocytosis (55,56). Liposomes and micelles have also been reported to accumulate in the ischemic myocardium via the EPR effect (34–36,38). It was also found that liposomes could cross the undamaged wall of myocardial capillaries in the isolated heart through endocytosis (57).

The results we have obtained with CoQ10-L have expected similarity with the results earlier obtained by us with ATP-loaded liposomes (38,39). Although each in its own way, both CoQ10 and ATP can assist hypoxic cells in maintaining

improved energy status. The tempting idea of using combinations of these two preparations for minimizing the extent of ischemic damage is currently under investigation in our laboratory.

In conclusion, CoQ10-L can effectively protect the myocardium from ischemia/reperfusion damage by diminishing the size of the irreversibly damaged zone in rabbits with an acute experimental myocardial infarction. CoQ10-L could provide an effective exogenous source of the CoQ10 *in vivo* and serve as a tool for cardiomyocyte protection in ischemically damaged hearts suffering from the reduced CoQ10 supply during an ischemic attack although, the exact application set up may be quite different from ours.

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