Vitamin E Deficiency Fails to Affect Myocardial Performance During *In Vivo* Ischemia-Reperfusion

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Abstract: Vitamin E content, of cardiac tissue, has been proposed to play a major, role in the damage caused by myocardial ischemia-reperfusion (I-R). Previous studies using *in vitro* models have examined vitamin E deficiency and I-R-induced myocardial damage with equivocal results. The purpose of this study was to use an *in vivo* model of myocardial I-R to determine the effects of vitamin E deficiency on myocardial I-R-induced damage. Female Sprague-Dawly rats (4-mo old) were assigned to either: 1) control diet (CON), or 2) vitamin E deficient diet (VE-DEF). The CON diet was prepared to meet AIN-93M standards, which contains 75 IU vitamin E/kg diet. The VE-DEF diet was the AIN-93M diet prepared with tocopherol stripped corn oil and no vitamin E. Following a 14-week feeding period, significant differences ($p < 0.05$) existed in mean myocardial VE levels between groups (mean values ± SEM: CON = 48.2 ± 3.5; VE-DEF = 12.4 ± 1,4 μg VE/g wet weight). Animals from both experimental groups were subjected to an *in vivo* I-R protocol consisting of 25 minutes of left coronary artery occlusion followed by 10 minutes of reperfusion. No group differences ($p > 0.05$) existed in cardiac performance (peak arterial pressure or ventricular work) or the incidence of ventricular arrhythmias during the I-R protocol. VE-DEF animals had significantly higher ($p < 0.05$) levels of myocardial lipid peroxidation and lower ($p < 0.05$) protein thiols following I-R compared to the CON animals. These data suggest that although vitamin E deficiency increases oxidative damage resulting from myocardial I-R, it does not affect cardiac performance during the insult.

Key words: Vitamin E deficiency, myocardial ischemia-reperfusion, free radicals, lipid peroxidation, ventricular arrhythmias, cardiac performance

Introduction

There is much interest in nutritional antioxidants as a therapeutic preventative strategy against myocardial ischemia-reperfusion (I-R) injury. The interest results from findings that reactive oxygen species (ROS) contribute significantly to myocardial I-R injury [1]. ROS such as superoxide anions, hydroxyl radicals and peroxyl radicals

formed during reperfusion [2, 3], can lead to lipid peroxidation within the cardiac myocyte, resulting in decreased myocardial performance [4]. Under normal oxidative conditions the cell is protected from ROS-induced damage by a number of antioxidants, of which vitamin E (VE) is thought to be the primary lipid soluble antioxidant [5]. Accordingly, myocardial VE content could be a key factor for protecting the heart against I-R injury.

To date, all of the investigations examining the effects of VE on the heart have manipulated the VE content of the tissue and examined myocardial contractile performance and injury using *in vitro* models of I-R. Unfortunately, the results of these studies are equivocal with some investigators reporting that VE deficiency is detrimental to the heart during an I-R insult [6–13], whereas other studies indicate that myocardial VE levels do not influence the cardiac response to I-R [14, 15]. For example, myocardial VE deficiency has been reported to result in increases in I-R-induced lipid peroxidation [6–10], creatine kinase efflux [11, 12] and the incidence of reperfusion-induced arrhythmias [13].

In contrast, several studies have reported that VE deficiency does not effect myocardial I-R injury or cardiac performance. For example, Shuter *et al* [14] reported that rats fed a VE deficient diet had no reperfusion-induced arrhythmias or decrement in myocardial performance during I-R. In support of this, O'Farrell and Jackson [15] found no significant differences in myocardial creatine kinase release between VE deficient and supplemented hearts subjected to *in vitro* I-R. The explanation of these discrepant findings is unclear and additional research is required to resolve this conflict.

Although the *in vitro* model of myocardial I-R has the advantage of studying the heart during carefully controlled preload and afterload conditions, this paradigm does not mimic *in vivo* conditions associated with myocardial I-R. Surgical occlusion of the left anterior descending coronary artery in open-chest anesthetized rats an *in vivo* model that has been widely used as a more physiologically relevant model for studying myocardial I-R [16–20]. This model allows examination of the myocardium while systemic changes, such as catecholamine release, are occurring thus providing a more representative environment to study the effects of various nutritional strategies on myocardial I-R damage. The purpose of this study was to investigate the effects of VE deficiency on myocardial performance and biochemical damage resulting from an I-R insult. Based on previous work from our lab which indicated that dietary supplementation with VE decreased I-R-induced myocardial oxidative damage but failed to affect cardiac performance [16], we hypothesized that nutritional VE deficiency would increase markers of oxidative injury but have no effect on cardiac contractile performance during *in vivo* I-R.

Materials and Methods

Animals and dietary supplementation: Female Sprague Dawley rats (4-mo old) were assigned to one of two dietary groups; 1) control (CON), n = 12; or 2) vitamin E deficient (VE-DEF), n = 18. CON animals were fed the AIN-93M purified diet containing 75 IU/kg diet dl-alpha-tocopheryl acetate. VE-DEF animals consumed the AIN-93M purified diet prepared with tocopherol stripped corn oil and no added VE. The animals were fed for 14-weeks. Diets were professionally prepared by Harlan Teklad Inc., Madison, WI. To ensure that all animals consumed equal amounts of their respective diets, animals in the VE-DEF were pair fed with CON animals. This ensured similar caloric consumption and weight gain across both groups. Randomly selected animals from the two dietary groups were exposed to the I-R protocol. The remaining animals from each group served as sham controls by undergoing the same surgical interventions without I-R. The hearts from the sham animals provided baseline data for levels of oxidative damage and endogenous antioxidants.

Experimental protocol: Upon completion of the feeding period, animals were anesthetized with 40 $mg \cdot kg^{-1}$ sodium pentobarbital and ventilated with room air (tidal volume = 0.7 ml/100 g body weight, frequency = 80 respirations/min). Rectal temperature was monitored and body temperature was maintained at 37°C with a heating blanket. Electrical activity of the heart was determined using a standard limb (lead II) ECG. The chest was opened via a left thoracotomy and a ligature was placed around the left coronary anterior descending (LCAD) artery close to its origin. In the I-R surgery animals, coronary occlusion was achieved by passing both ends of the ligature through a small plastic tube, which was then pressed on the surface of the heart directly above the coronary artery. The resulting arterial occlusion was maintained for 25 minutes by clamping the plastic tube and ligature with small hemostats. Reperfusion was achieved by removing the hemostats and tube. Cardiac performance and ECG was monitored during ischemia and for the 10-min reperfusion period after which the heart was removed and rapidly frozen in liquid nitrogen for subsequent biochemical analyses.

Validation of coronary occlusion and reperfusion: The aforementioned technique of coronary occlusion has been used successfully by our group [16–18] and other laboratories [19, 20]. To validate that coronary occlusion was achieved, we performed preliminary experiments where Evans blue dye was injected directly into the right ventricle cavity (i.e. upstream from the ligature). After injection of the dye, the heart was removed within 10 seconds and

examined for evidence of dye in the ventricular mass supplied by the LCAD. Failure to observe dye stain in this area of the ventricle was interpreted as achievement of coronary occlusion.

To ensure that reperfusion had been adequately achieved in these preliminary studies, we also administered Evans blue dye at the end of the 10-minute reperfusion period. In each case we observed a uniformly stained heart, which was interpreted as evidence that reperfusion was achieved.

Evaluation of rhythm disturbances: Standard limb lead (lead II) ECG recordings were obtained using a high-speed recorder (Grass Instruments Polygraph, model 79) throughout the protocol. They were independently analyzed by two investigators according to the Lambeth conventions [21] for the incidence and total number or time of: 1) premature ventricular beats; 2) ventricular fibrillation; and 3) ventricular tachycardia. If discrepancies existed between the two investigators, a third investigator was used to arbitrate.

Measurement of cardiac contractile function: To monitor cardiovascular function, arterial pressure was measured by placing a fluid filled catheter into the ascending aorta via the carotid artery using techniques described by Geenen *et al* [22]. The catheter was connected to a miniature pressure transducer and interfaced with a computerized heart performance analysis system (Digi-Med, Louisville, Kentucky). Cardiac function measurements (peak arterial pressure, total ventricular work [heart rate $\times$ peak arterial pressure]) were performed prior to ischemia and continued during the I-R protocol. A primary factor in our decision not to measure ventricular pressures directly was based on the observation that catheters placed in the left ventricles of rats promote catheter-induced ventricular arrhythmias. This observation was seen to be meaningful because ventricular arrhythmias were considered to be an important dependent variable in this investigation.

Vitamin E determination: Myocardial levels of VE were determined with high performance liquid chromatography using the protocol of Cort *et al* [23]. Left ventricular samples were homogenized in acetone using a mechanical homogenizer (Ultra-Turrax T25, IKA Works, Cincinnati, Ohio). Samples were extracted twice with petroleum ether, then reconstituted in isooctane and analyzed for VE content. The isooctane extract (20 μL) was injected onto a 250 $\times$ 4 mm, 10 micron LiChrosorb SI column (Baird and Tatlock, Dagenham, UK).

Lipid peroxidation measurements: To determine the amount of oxidative damage in the heart, left ventricular levels of lipid peroxidation were measured using two methods. First, malondialdehyde (MDA) levels were measured spectrophotometrically using the thiobarbituric acid-reactive substance (TBARS) method described by Uchiyama and Mihara [24]. Secondly, lipid hydroperoxides were quantified using the ferrous oxidation/xylenol orange technique described by Hermes-Lima *et al* [25]. Cumene hydroperoxide was used as the standard for this assay and hydroperoxide values are expressed in cumene hydroperoxide equivalents (CHE). In our hands, the coefficients of variation for the TBARS and the lipid hydroperoxide assays are ~3 and 4 percent respectively.

Determination of tissue thiol content: Tissue thiols are important in the regulation of both cellular redox status and antioxidant capacity. To further determine the degree of oxidative damage, total, protein and non-protein thiols were assayed from the left ventricle of all experimental animals using the DTNB-based spectrophotometric technique described by Jocelyn [26].

Determination of antioxidant enzyme activity: To determine of our dietary treatments altered endogenous antioxidant enzyme activity, a small sample of the left ventricle from each animal was assayed for total superoxide dismutase (SOD), the copper-zinc isoform of SOD (Cu-Zn SOD), the manganese isoform of SOD (MnSOD), glutathione peroxidase (GPX), and catalase (CAT) activities. SOD and GPX activities were determined using a modification of the procedures described by Oyanagui [27], and Flohe and Gunzler [28], respectively. Catalase was assayed using the procedure described by Aebi [29] and modified by Ji *et al* [30].

Statistical analysis: Biochemical measurements and arrhythmia data were subjected to a one-way analysis of variance. Myocardial performance was analyzed using a two-way repeated measures analysis of variance. A Scheffe test was used *post-hoc* to determine where differences existed. Significance was established at $p < 0.05$.

Results

Figure 1 contains the mean (± SEM) myocardial VE concentrations in both experimental groups. Myocardial VE levels in the VE-DEF animals were significantly lower ($p < 0.05$) than CON animals. Compared to sham, I-R did not alter ($p > 0.05$) myocardial VE levels in either experimental group.

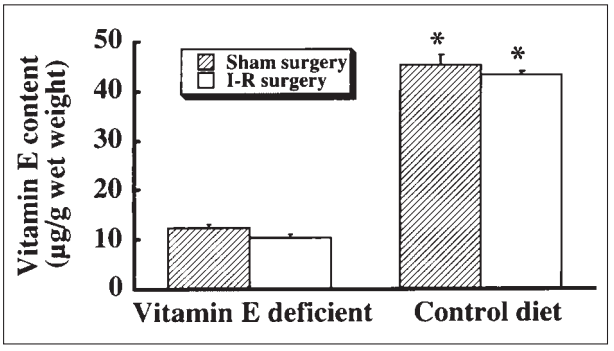

Figure 1: Vitamin E levels in the myocardium of CON and VE-DEF rats undergoing either sham or I-R surgery. * Different from VE-DEF animals with same surgical treatment ($p < 0.05$). Values are means (± SEM).

Two markers of lipid peroxidation were used to determine the effects of the different diets on cardiac damage due to I-R. Figure 2 contains the mean (± SEM) values for myocardial TBARS and cumene hydroperoxide equivalents (CHE) in both experimental groups. Note that the directions of these two markers of lipid peroxidation are in agreement between groups. In this record, no significant differences ($p > 0.05$) in MDA and CHE existed between VE-DEF sham animals compared to CON sham animals. A key finding was that MDA and CHE levels were significantly greater ($p < 0.05$) in VE-DEF I-R surgery animals compared to the CON I-R surgery animals. This indicates a greater degree of I-R induced lipid peroxidation in VE-DEF animals.

The effects of dietary treatments on cardiac contractile function are summarized in Figure 3. Two markers of contractile function were used: 1) peak arterial pressure and 2) total ventricular work (estimated as heart rate × peak arterial pressure). No difference existed ($p > 0.05$) between groups in these variables.

Table I contains the number and incidence of ventricular premature beats (VPB), ventricular tachycardia and mortality during I-R in both experimental groups. No significant differences ($p > 0.05$) existed in arrhythmias between groups during either ischemia or reperfusion.

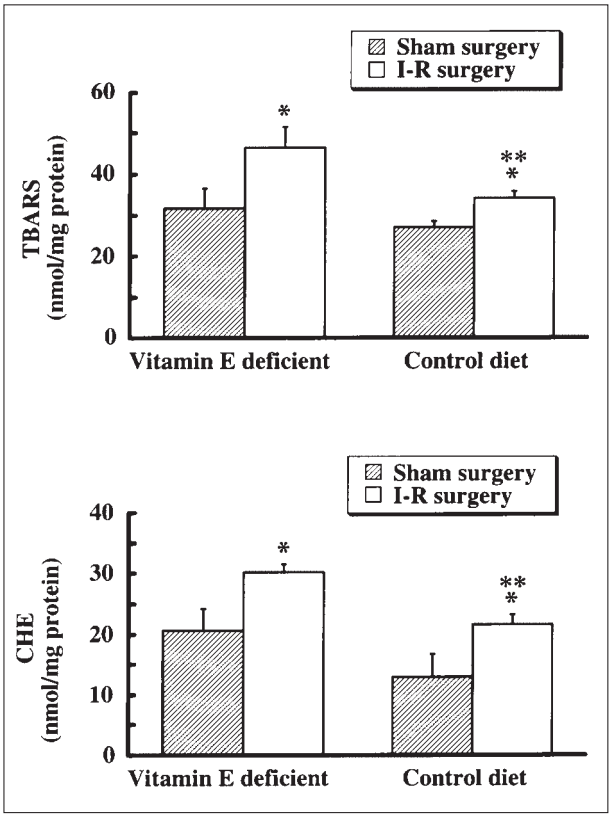

Figure 2: TBARS and CHE concentrations in the myocardium of CON and VE-DEF rats undergoing either sham or I-R surgery. * Different from sham surgery with same dietary treatment ($p < 0.05$). ** Different from VE-DEF animals that underwent I-R surgery ($p < 0.05$). Values are means (± SEM).

Mean (± SEM) myocardial levels of total thiols, protein thiols, and non-protein thiols are contained in Figure 4. Animals exposed to I-R contained lower ($p < 0.05$) total thiols in both CON and VE-DEF groups suggesting that oxidation of cardiac tissue sulfhydryl groups uccurred during I-R. In the VE-DEF I-R animals the decrease in total thiols was due to a loss in both protein and non-protein thiols, however in the CON I-R animals the decrease in total thiols was only due to a loss in non-protein thiols.

Table I: Summary of cardiac arrhythmias evoked by ischemia and reperfusion. Values are group means ± SEM. No bigeminy ventricular ectopy was observed in any animal

	Vitamin E deficient diet (n = 10)		Control diet (n = 7)	
Category	Ischemia	Reperfusion	Ischemia	Reperfusion
Mean number of ventricular premature beats (VPB)	13.0 ± 6.0	0.2 ± 0.2	9.2 ± 5.5	0.8 ± 0.3
VPB (% of animals)	86	14	88	75
Mean number of salvos (> 2 consecutive VPB)	1.4 ± 1.3	0	0.1 ± 0.1	0
Ventricular tachycardia (seconds)	1.1 ± 0.8	0	0.2 ± 0.0	0.1 ± 0.1
Ventricular tachycardia (% of animals)	42	0	18	9

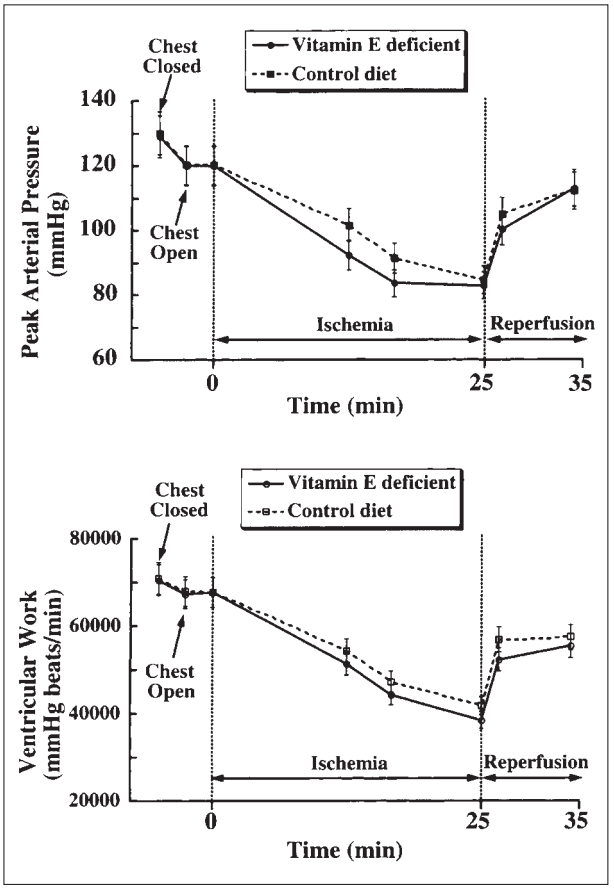

Figure 3: The effects of CON and VE-DEF diets on cardiac contractile function: 1) peak arterial pressure and 2) total work performed which was estimated as the products of heart rate and peak arterial pressure. Values are means (± SEM). No differences existed ($p > 0.05$) in any of these variables between groups.

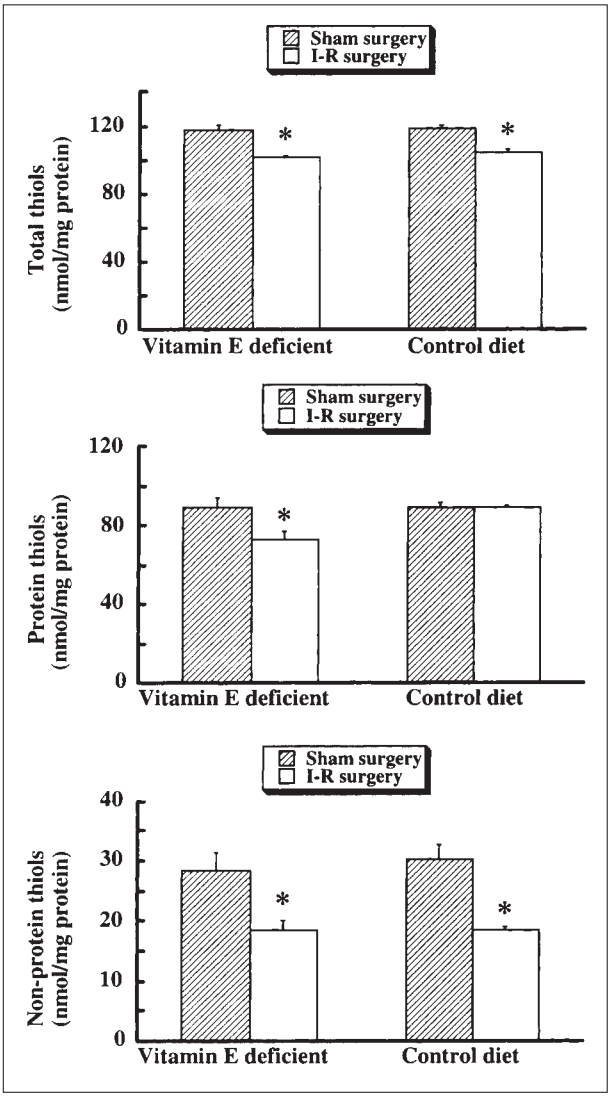

Figure 4: Thiol concentrations in the myocardium of CON and VE-DEF rats undergoing either sham or I-R surgery. * Different from sham surgery ($p < 0.05$). Values are means (± SEM).

Table II contains mean (± SEM) left ventricular activities of GPX, SOD (both the copper-zinc [Cu-Zn] and manganese [Mn] dependent isoforms) and CAT. There were no differences in basal levels of any of the antioxidant enyzme activities between groups. However, GPX, Cu-Zn SOD and total SOD activity were significantly ($p < 0.05$) higher in the VE-DEF hearts following I-R compared to the VE-DEF sham-treated hearts.

Table II: Effects of vitamin E deficiency on antioxidant enzyme activities. Values are group means ± SEM

	Vitamin E deficient diet		Control diet	
Antioxidant Enzyme	Sham surgery	I-R surgery	Sham surgery	I-R surgery
Glutathione peroxidase (μmol/min/mg protein)	1.76 ± 0.23	2.26 ± 0.32*	1.81 ± 0.54	1.90 ± 0.77
Total Superoxide dismutase (Units/mg protein)	37.6 ± 1.1	41.2 ± 1.2*	37.7 ± 1.3	38.2 ± 1.1
Cu-Zn Superoxide dismutase	18.9 ± 0.7	22.3 ± 1.0*	21.1 ± 1.2	20.4 ± 0.8
Mn Superoxide dismutase (Units/mg protein)	18.7 ± 1.6	18.9 ± 0.5	16.1 ± 1.8	17.2 ± 1.3
Catalase (Units/mg protein)	1.51 ± 0.07	1.61 ± 0.07	1.37 ± 0.24	1.51 ± 0.11

* different from sham surgery animals with the same diet ($p < 0.05$)

Discussion

Overview of principle findings: This was the first study to use an *in vivo* model of myocardial I-R to examine the effects of VE deficiency on cardiac injury and performance. Our findings support the hypothesis that nutritional VE deficiency increases I-R induced myocardial lipid peroxidation but does not impact cardiac performance during *in vivo* I-R.

Vitamin E deficiency fails to influence cardiac performance or ventricular arrhythmias during I-R: Compared to CON, myocardial VE levels were 75% lower in the VE-DEF animals, however this VE deficiency had no significant effect on myocardial contractile performance (peak arterial pressure, ventricular work) or ventricular arrhythmias during *in vivo* I-R. These results agree with the only other known study that has examined the effects of VE deficiency on myocardial contractile performance during I-R [14]. Specifically, Shuter *et al* [14] fed rats a VE deficient diet for nine weeks, which decreased myocardial VE content by 70%. Hearts isolated from the VE deficient rats were subjected to global ischemia and subsequent reoxygenation *in vitro*. Compared to control diet fed animals, hearts from VE deficient animals did not differ in the number of ventricular arrhythmias or cardiac performance, as measured by cardiac output and +dP/dt. Therefore, based on both *in vitro* and the present *in vivo* study, it appears that myocardial VE deficiency does not affect cardiac performance during I-R.

There are a number of potential explanations why nutritional VE deficiency has no influence on cardiac performance during myocardial I-R. First, it is difficult to deplete the myocardium of VE; thus the remaining low concentration of VE may be sufficient to protect against ROS mediated damage. In support of this notion, Marchant *et al* [11] demonstrated that only small amounts of myocardial VE are required to provide ROS scavenging capacity.

A second possibility is that the ROS generated in the lipid bilayer do not play a significant role in modifying the contractile function of the myocardium. Perhaps mild to moderate lipid peroxidation is not an important factor in the pathogenesis of acute myocardial dysfunction and that exacerbating this damage by feeding animals a VE deficient diet does not cause enough damage to result in cardiac dysfunction.

A final explanation for the finding that VE deficiency had no effect on cardiac performance during *in vivo* I-R is that a diet low in VE may be associated with an increase in the activity of other antioxidants that compensate for the decreased VE. This notion is supported by the data in Table II, which indicate an increase in myocardial GPX and SOD activity in VE-DEF animals after exposure to I-R. The increase in total SOD activity as due to an increase in the Cu-Zn SOD isoform activity. Walsh *et al* [31] reported that myocardial antioxidant enyzme activity increased in rats fed a VE deficient diet when hearts were subjected to *in vitro* peroxidative challenge. These authors conclude that the adpatation was an attempt by the myocardial cell to mitigate the effects of the oxidative stress. Another antioxidant that may have played a role in the protection is vitamin C. Although the rat synthesises vitamin C, supplementation with this nutrient has been shown to improve myocardial performance in the isolated perfused working heart model using hearts from diabetes-induced rats [32]. Unfortunately vitamin C was not measured in this study therefore the role that it may have played in the protection can not be determined.

The finding that nutritional VE deficiency failed to influence the number or incidence of ventricular arrhythmias during myocardial I-R is contrary to a previous *in vitro* study [13]. These authors reported that hearts from VE deficient rats made globally ischemic generated significantly more arrhythmias during reperfusion compared to hearts from rats fed a standard diet. Again, it appears that the findings from *in vitro* models of I-R may not always be indicative of *in vivo* responses.

Influence of vitamin E deficiency on biochemical alterations: An important finding in the current study was that following I-R, both MDA and CHE levels were significantly higher in VE-DEF animals compared to CON animals indicating greater I-R induced lipid peroxidation. These findings agree with work by previous authors who report an increase in lipid peroxidation using *in vitro* models of I-R [6–10] but contradict other studies reporting no evidence for an increase in MDA following I-R [9, 11]. VE deficient cells provide limited protection to the polyunsaturated fatty acids in the cell membranes making them more susceptible to oxidative damage in the membrane resulting in a loss of membrane fluidity and function [33]. Hence, it is predictable that VE-DEF tissue exposed to increased ROS production would experience greater lipid peroxidation.

Critique of the model: The adult female Sprague Dawley rat was chosen as the experimental model because: 1) the nature of these invasive experiments precludes the use of human subjects; 2) this rat is highly inbred and does not display large inter-animal variation in coronary collateral circulation [34]; 3) the rat is widely accepted model for the study of VE deficiency [8, 10, 11] and 4) adult animals were chosen as experimental subjects to avoid confounding variables associated with both development and old age.

The surgical procedure, including the time of occlusion

and reperfusion, used in these xperiments has been used successfully in our laboratory [16–18] and by others [19, 20] and has been shown to result in both myocardial ischemia and reperfusion. However, it is possible that this type of experimental surgery could result in inter-animal differences in either the magnitude of ischemia or reperfusion. Nonetheless, we believe that these differences are clinically relevant and better reflect the types of I-R insults that occur in humans.

Summary and conclusions: These data support the notion that VE deficiency increases myocardial lipid peroxidation following an *in vivo* I-R insult. However, these experiments do not support the hypothesis VE deficiency significantly compromises cardiac function during *in vivo* I-R. Importantly, the fact that our *in vivo* findings differ from previous *in vitro* experiments reinforces the notion that antioxidant experiments should be conducted in a physiological environment prior to reaching conclusions about the *in vivo* effects of the deficiency.

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