Journal of the American College of Cardiology
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Published by Elsevier Science Inc.

Vol. 38, No. 6, 2001
ISSN 0735-1097/01/$20.00
PII S0735-1097(01)01596-0

EXPERIMENTAL STUDIES

Antioxidant Vitamins Attenuate Oxidative Stress and Cardiac Dysfunction in Tachycardia-Induced Cardiomyopathy

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OBJECTIVES We administered antioxidant vitamins to rabbits with pacing-induced cardiomyopathy to assess whether antioxidant therapy retards the progression of congestive heart failure (CHF).

BACKGROUND Although oxidative stress is increased in CHF, whether progression of heart failure could be prevented or reduced by antioxidants is not known.

METHODS Rabbits with chronic cardiac pacing and sham operation were randomized to receive a combination of beta-carotene, ascorbic acid and alpha-tocopherol, alpha-tocopherol alone or placebo over eight weeks. Echocardiography was used to measure cardiac function weekly. Resting hemodynamics and in vivo myocardial beta-adrenergic responsiveness were studied at week 8. Animals were then sacrificed for measuring myocardial beta-receptor density, norepinephrine (NE) uptake-1 site density, sympathetic neuronal marker profiles, tissue-reduced glutathione/oxidized glutathione (GSH/GSSG) ratio and oxidative damage of mitochondrial DNA (mtDNA).

RESULTS Rapid cardiac pacing increased myocardial oxidative stress as evidenced by reduced myocardial GSH/GSSG ratio and increased oxidized mtDNA and produced cardiac dysfunction, beta-adrenergic subsensitivity, beta-receptor downregulation, diminished sympathetic neurotransmitter profiles and reduced NE uptake-1 carrier density. A combination of antioxidant vitamins reduced the myocardial oxidative stress, attenuated cardiac dysfunction and prevented myocardial beta-receptor downregulation and sympathetic nerve terminal dysfunction. Administration of alpha-tocopherol alone produced similar effects, but the effects were less marked than those produced by the three vitamins together. Vitamins produced no effects in sham-operated animals.

CONCLUSIONS Antioxidant vitamins reduced tissue oxidative stress in CHF and attenuated the associated cardiac dysfunction, beta-receptor downregulation and sympathetic nerve terminal abnormalities. The findings suggest that antioxidant therapy may be efficacious in human CHF. (J Am Coll Cardiol 2001;38:1734–40) © 2001 by the American College of Cardiology

Increased production of oxygen free radicals and decreased oxidant capacity occur in congestive heart failure (CHF) (1–4). This pro-oxidant shift in the intracellular redox state may induce cell death by either direct cell membrane damage by lipid peroxidation (5) or apoptosis through activation of transcription factors (6). These changes occur not only in cardiomyocytes but also in cardiac sympathetic nerves, which are very sensitive to oxidative damage (7).

The purpose of this study was to determine the functional importance of oxidative stress in animals with CHF by administering antioxidant vitamins (beta-carotene, ascorbic acid and alpha-tocopherol). Antioxidant vitamins have been shown to reduce oxidative stress in the heart produced by norepinephrine (NE) and prevent the NE-induced myocardial beta-adrenergic subsensitivity, myocyte apoptosis and noradrenergic nerve terminal dysfunction (8,9). To determine whether these vitamins also reduce oxidative stress and attenuate the deterioration of left ventricular (LV) mechanical function and cardiac noradrenergic nerve dysfunction in CHF (10,11) we carried out this study using a rapid cardiac pacing model, which is known to increase oxygen free radical production (12). We administered either a combination of antioxidant vitamins (beta-carotene, ascorbic acid and alpha-tocopherol) or alpha-tocopherol alone to animals with CHF and sham-operated animals for eight weeks. The results were compared with those of placebo-treated animals. We measured global cardiac function, myocardial beta-receptor density, NE uptake-1 site density and cardiac sympathetic nerve transmitter profiles. To assess the antioxidant effects of vitamins we measured cardiac tissue-reduced glutathione/oxidized glutathione (GSH/GSSG) ratio and mitochondrial DNA (mtDNA) 8-oxo-7,8-

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Manuscript received March 30, 2001; revised manuscript received July 17, 2001, accepted August 13, 2001.

Abbreviations and Acronyms

CHF	= congestive heart failure
dP/dt	= first derivative of LV pressure
dG	= 2′-deoxyguanosine
EDD	= end-diastolic dimension
ESD	= end-systolic dimension
FS	= fractional shortening
GSH	= reduced glutathione
GSSG	= oxidized glutathione
LV	= left ventricular
MI	= myocardial infarction
mtDNA	= mitochondrial DNA
NE	= norepinephrine
8-oxo-dG	= 8-oxo-7,8-dihydro-2′-deoxyguanosine

dihydro-2′-deoxyguanosine (8-oxo-dG), a sensitive, stable marker of oxidative stress in cellular DNA (13).

METHODS

Experimental model. This study was approved by the University of Rochester Committee on Animal Resources and conformed to the guiding principles approved by the Council of the American Physiological Society and National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHHS Publication No. [NIH] 85-23, Revised 1985, Office of Science and Health Reports).

Congestive heart failure was induced in adult New Zealand White rabbits (2.8 to 3.6 kg) using a modified rapid cardiac pacing technique (11). Briefly, subxyphoid thoracotomy and pericardiotomy were performed under isoflurane gas anesthesia. Two shielded pacing leads (TPW50, Ethicon, Inc., Somerville, New Jersey) were sutured onto the LV apex and left pectoral muscle, respectively, and exteriorized to the interscapular region. One week later, animals were randomly assigned to receive pacing at a rate of 360 beats/min with a modified implantable programmable pacemaker (Model 8086 Prevail, Medtronic Inc., Minneapolis, Minnesota) (animals with CHF) or no cardiac pacing (sham animals).

Experimental protocol. The animals with CHF and sham animals were each divided into three groups according to the vitamin regimens: 1) beta-carotene, 20 mg; ascorbic acid, 200 mg and alpha-tocopherol, 200 mg; 2) alpha-tocopherol, 200 mg; and 3) placebo. The doses were designed to infuse evenly over eight weeks using subcutaneous pellets (Innovative Research of America, Sarasota, Florida). Animals were examined for clinical evidence of heart failure and echocardiographic changes of LV mechanical function. After eight weeks of cardiac pacing, pacing was discontinued, and the animals were anesthetized for measuring arterial blood NE, resting hemodynamics and cardiac inotropic responses to isoproterenol. The animals were then sacrificed with intravenous sodium pentobarbital (>100 mg/kg). The heart was removed and weighed and rinsed in ice-cold oxygenated normal saline. Fresh LV free wall was taken for measuring glutathione and noradrenergic nerve terminal transmitter profiles by NE histofluorescence and tyrosine hydroxylase immunocytochemistry. Remaining LV tissue was stored in liquid nitrogen for later measurements of myocardial NE uptake-1 carrier sites, beta-adrenoceptor density and mtDNA 8-oxo-dG.

Echocardiographic and hemodynamic measurements. Two-dimensional and M-mode echocardiography were obtained using a 5-MHz transducer on a Toshiba Model SSH-60A sonographic system (Toshiba America Medical Systems, Tustin, California). Maximal LV end-diastolic dimension (EDD) and end-systolic dimension (ESD) were measured and used to calculate LV fractional shortening (FS) by the following equation: FS = [(EDD − ESD)/EDD × 100].

For the hemodynamic studies, the animals were anesthetized with ketamine (35 mg/kg) and midazolam (0.8 mg/kg). A 20-gauge fluid-filled catheter (Insyte, Deseret Medical Inc., Becton, Dickinson and Company, Sandy, Utah) was inserted into the left carotid artery for measuring aortic pressure, while a 2F micromanometer-tipped catheter (Millar Instruments Inc., Houston, Texas) was advanced into the left ventricle via the right carotid artery for measuring the LV pressures. Electrocardiograms, aortic pressure and the first derivative of LV pressure (dP/dt) were recorded on a multichannel recorder (Brush Model 480, Gould Inc., Instrument Systems Division, Cleveland, Ohio). At least 1 h was allowed to elapse after the catheterization before the resting hemodynamic data were taken in triplicate over a 15 to 20 min steady state. The measurements were averaged and used for statistical analyses. Two doses of isoproterenol (0.4 and 0.8 μg/kg, intravenous) were then administered to measure the peak LV dP/dt response to beta-agonist stimulation. Five consecutive beats at either baseline or peak response were averaged for statistical analyses.

Myocardial glutathione measurement. Fresh LV myocardium was homogenized in three volumes of 1% picric acid and the supernatant collected for measuring total glutathione using a glutathione reductase-coupled enzymatic assay (14) and a Perkin Elmer Lambda 3 UV/VIS spectrophotometer (Perkin Elmer, Norwalk, Connecticut). Oxidized glutathione was measured by masking the GSH by 2-vinyl pyridine in the enzymatic assay. The ratio of GSH to GSSG is a measure of total tissue oxidative stress.

Myocardial mtDNA 8-oxo-dG measurement. Left ventricular muscle samples were prepared for isolation of mitochondria (15). The mtDNA was extracted using QIAamp blood kit (Qiagen, Inc., Valencia, California) and injected into a YMC Basic S 3 μ column (4.6 × 150 mm, Waters Corp., Milford, Massachusetts) in a BAS 480 HPLC (Bioanalytical Systems, Inc., West Lafayette, Indiana) for measuring 8-oxo-dG and 2′-deoxyguanosine (dG). A Model 5200A Coulochem II electrochemical detector equipped with a Model 5011 analytical cell and Model 5021

Table 1. Weights and Resting Hemodynamics in Sham and Pacing-Induced Rabbits With Heart Failure

	Sham		CHF		
	Placebo	Vitamins	Placebo	Vitamins	Vitamin E
Number of animals	11	10	10	10	8
BW (kg)	3.1 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.1 ± 0.1	3.1 ± 0.1
HW (g)	7.1 ± 0.3	6.3 ± 0.2	7.8 ± 0.3	7.3 ± 0.3	7.9 ± 0.2
HR (beats/min)	257 ± 9	264 ± 8	251 ± 11	248 ± 11	253 ± 7
MABP (mm Hg)	98 ± 3	97 ± 3	86 ± 3*	98 ± 5	95 ± 3
RAP (mm Hg)	2.0 ± 0.4	2.2 ± 0.2	6.1 ± 0.8*	5.3 ± 0.6*	5.5 ± 0.6*
LVEDP (mm Hg)	7.2 ± 0.6	7.7 ± 1.1	24.2 ± 1.9*	19.4 ± 1.5*	23.3 ± 1.8*
LVdP/dt (mm Hg/s)	3,530 ± 393	3,650 ± 273	2,010 ± 165*	2,685 ± 181†	2,445 ± 375
Plasma (NE) (pg/ml)	126 ± 14	136 ± 17	391 ± 26*	321 ± 11*	348 ± 25*

*$p < 0.05$ compared with the Sham placebo group; †$p < 0.05$ compared with the CHF placebo group. Values are expressed as means ± SEM.

BW = body weight; CHF = congestive heart failure; HR = heart rate; HW = heart weight; LVEDP = left ventricular end-diastolic pressure; LVdP/dt = peak left ventricular dP/dt; MABP = mean aortic blood pressure; NE = norepinephrine; RAP = right atrial pressure.

guard cell (ESA, Inc., Chelmsford, Massachusetts) was used (16).

Myocardial beta-adrenoceptor density and NE uptake-1 carrier site density. Left ventricular beta-adrenoceptor density and NE uptake-1 carrier site density were measured by the radioligand binding technique using [^{125}I]-iodocyanopindolol (17) and [^{3}H] nisoxetine (11) (NEN Life Science Products, Inc., Boston, Massachusetts), respectively. The radioactivity was counted in a Tri-Carb 2400 TR liquid scintillation spectrometer (Packard Instrument, Downers Grove, Illinois). The number of receptor binding sites and dissociation constants were calculated using the EBDA computer software program (Elsevier Science, Cambridge, United Kingdom) (18).

Cardiac sympathetic nerve terminal neurotransmitter profiles. Cardiac sympathetic nerve profiles were assessed by histofluorescence for catecholamines and immunocytochemistry for tyrosine hydroxylase using sucrose-potassium phosphate-glyoxylic acid and sheep anti-tyrosine hydroxylase primary antibody, respectively (10). Tissue sections were photographed onto 35-mm slides with ×30 magnification. The numbers of catecholaminergic profiles and tyrosine hydroxylase profiles were counted morphometrically in 0.003536 mm^3 and 0.00885 mm^3 fields, respectively. At least six fields were counted for each animal and the results averaged.

Statistical analysis. Results are expressed as means ± SEM. Student *t* test was used to determine the difference between the sham rabbits and the rabbits with CHF. Analysis of variance (ANOVA) and post hoc Tukey comparison test was used to determine the statistical significance of differences among the three groups with CHF (treated with the three vitamin combination, alpha-tocopherol alone or placebo) and sham animals. For the serial echocardiographic data, ANOVA for repeated measures was used to determine the effects of pacing and vitamin treatment. A probability value of <0.05 was considered significant.

RESULTS

Clinical manifestations of CHF and resting hemodynamics. Body weight, heart weight and resting hemodynamics are shown in Table 1. In the sham-operated animals, there were no differences in any of the parameters between the placebo- and vitamin-treated groups. Chronic rapid cardiac pacing increased right atrial pressure and LV end-diastolic pressure and decreased mean aortic pressure and LV dP/dt. Animals with CHF exhibited decreased mobility and fluid retention (pleural effusion: 1.5 ± 0.4 ml; ascites: 10.3 ± 4.3 ml). There was a small, though statistically insignificant, increase in heart weight in animals with CHF compared with the sham animals. Administration of three antioxidant vitamins had no effects on body weight, heart rate, mean aortic pressure, right atrial pressure or LV end-diastolic pressure but improved resting LV dP/dt in animals with CHF. A qualitatively similar change occurred in LV dP/dt after alpha-tocopherol treatment alone, but the change was smaller than that produced by three antioxidant vitamins and did not reach statistical significance compared with the placebo-treated animals with CHF. Table 1 also shows that arterial NE concentration was increased in animals with CHF. Administration of vitamins did not affect arterial NE concentration in either sham animals or animals with CHF.

Serial echocardiographic changes in developing CHF. Figure 1 shows that rapid cardiac pacing caused a progressive increase in LV EDD ($F = 41.56$, $p < 0.001$) and a decrease in LV FS ($F = 25.26$, $p < 0.0001$) and that administration of antioxidant vitamins attenuated the increase of EDD and a decrease of FS in animals with CHF (triple vitamins: LVEDD: $F = 5.46$, $p < 0.0001$, FS: $F = 4.45$, $p < 0.0001$; alpha-tocopherol: LVEDD: $F = 2.28$, $p = 0.026$, FS: $F = 2.74$, $p = 0.008$). Compared to triple vitamins, vitamin E alone produced a smaller effect on FS in animals with CHF ($F = 3.75$, $p = 0.001$).

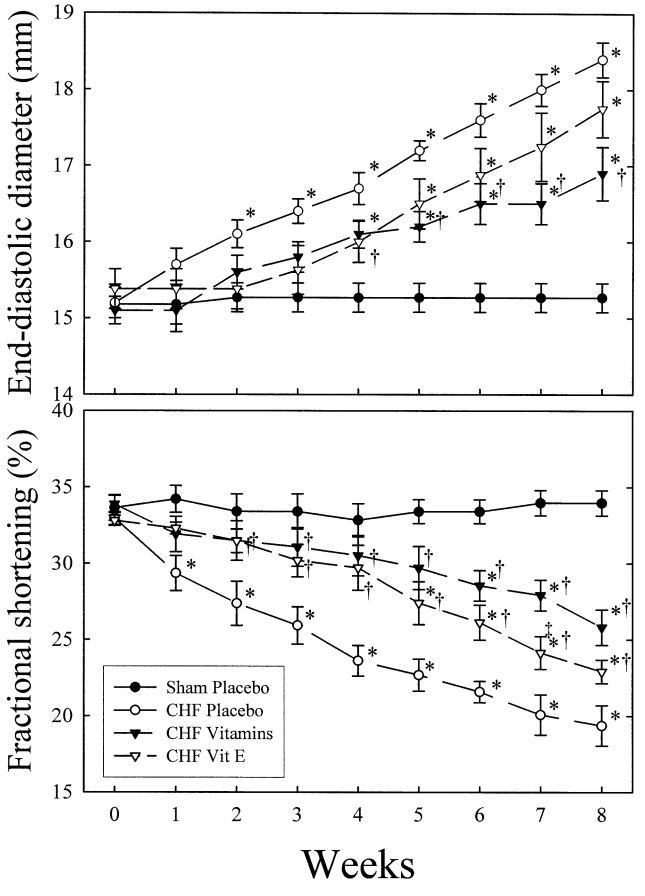

Figure 1. Serial changes in left ventricular end-diastolic dimension and fractional shortening in animals with congestive heart failure (CHF) and sham-operated animals. The changes in sham-operated animals treated with antioxidant vitamins were the same as in placebo-treated sham animals. **Bars** = SEM. *$p < 0.05$ vs. the sham animals; †$p < 0.05$ vs. the CHF + placebo group; ‡$p < 0.05$ vs. the CHF + vitamins group. The number of animals in each group is shown in Table 1.

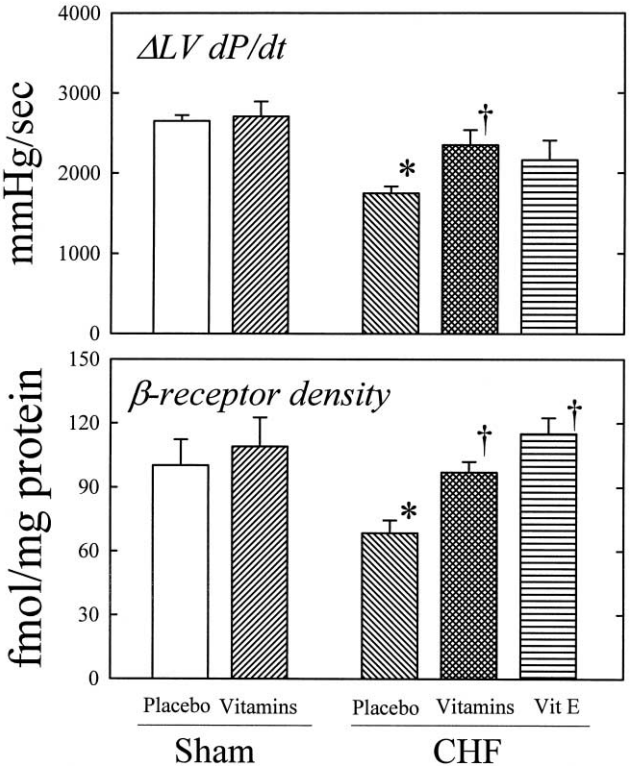

Figure 2. Change in left ventricular (LV) peak positive first derivative of LV pressure (dP/dt) in response to isoproterenol administration and myocardial beta-receptor density in sham animals and animals with congestive heart failure (CHF). Symbols as in Figure 1.

LV dP/dt responses to isoproterenol and myocardial beta-receptor density. Figure 2 shows the changes in peak LV dP/dt response produced by isoproterenol and myocardial beta-receptor density in sham animals and in animals with CHF. Isoproterenol produced a marked increase in LV dP/dt in sham animals. This increase was reduced in animals with CHF but restored in the animals treated with the multivitamins. Alpha-tocopherol alone also improved the LV dP/dt response to isoproterenol, but the magnitude of effect was smaller than that achieved by the three vitamins. The difference in the LV dP/dt response between the CHF placebo and alpha-tocopherol groups was not statistically significant.

Figure 2 also shows that rapid cardiac pacing-reduced myocardial beta-receptor density and myocardial beta-receptor downregulation in CHF was abolished by the administration of either three antioxidant vitamins or alpha-tocopherol alone. There were no differences in dissociation constant among the groups.

Myocardial glutathione and mtDNA 8-oxo-dG. Rapid cardiac pacing increased myocardial GSSG (0.13 ± 0.02 μmol/g vs. 0.02 ± 0.01 μmol/g in sham animals, $t = 5.74$, $p < 0.001$) and reduced the ratio of GSH to GSSG ([GSH]/[GSSG]) (Fig. 3). It also increased mtDNA 8-oxo-dG (9.0 ± 1.4 pg/g vs. 3.9 ± 0.8 pg/g in sham animals, $t = 3.18$, $p < 0.01$). Since there was no change in mtDNA dG, the ratio of mtDNA 8-oxo-dG to dG was also increased in animals with CHF (Fig. 3). Administration of beta-carotene, ascorbic acid and alpha-tocopherol had no effects in sham animals but abolished the changes in myocardial GSH/GSSG ratio and mtDNA 8-oxo-dG/dG. Administration of alpha-tocopherol also abolished the increase in mtDNA 8-oxo-dG/dG, but its effect on myocardial GSH/GSSG ratio was less marked than that produced by the three vitamins together.

Cardiac sympathetic nerve terminal profiles. Figure 4 shows that rapid cardiac pacing reduced cardiac NE uptake-1 carrier site density and catecholaminergic and tyrosine hydroxylase immunostained profiles. These changes in CHF were attenuated by the administration of beta-carotene, ascorbic acid and alpha-tocopherol. In contrast, antioxidant vitamins had no effects on the sympathetic nerve terminal profiles in sham animals. Furthermore, although administration of alpha-tocopherol alone produced qualitatively similar results in animals with CHF, the magnitude of changes was smaller than those produced by the three vitamins together.

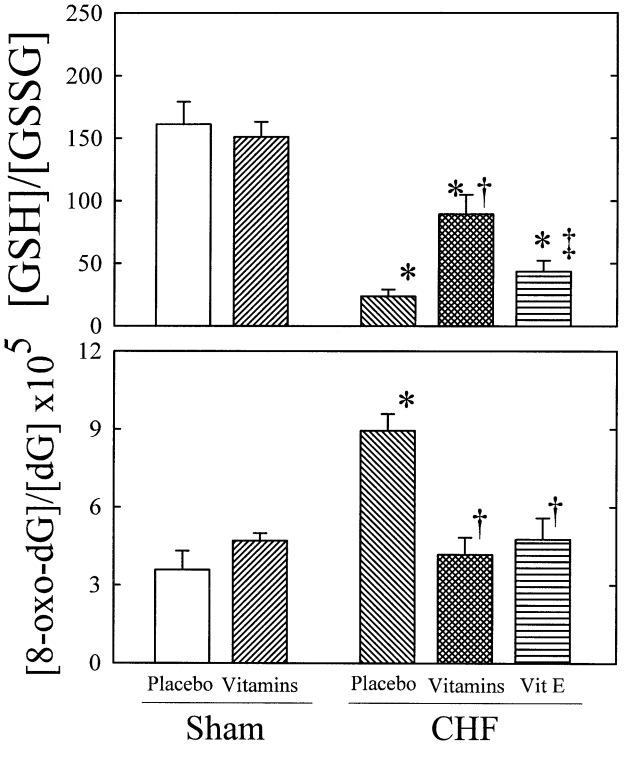

Figure 3. Changes in myocardial reduced glutathione/oxidized glutathione (GSH/GSSG) and mitochondrial DNA (mtDNA) 8-oxo-7,8-dihydro-2′-deoxyguanosine/2′-deoxyguanosine (8-oxo-dG/dG) ratios in sham animals and animals with CHF. Symbols as in Figure 1.

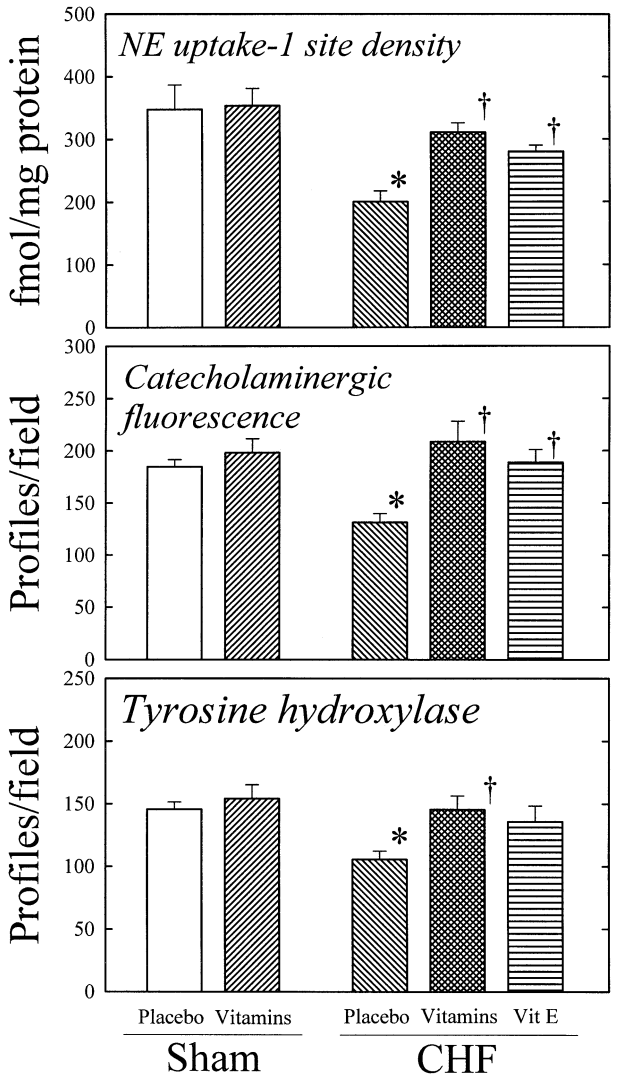

Figure 4. Changes in left ventricular norepinephrine (NE) uptake-1 carrier site density, catecholaminergic fluorescence profile and tyrosine hydroxylase immunostained profile in sham animals and in animals with congestive heart failure (CHF). Symbols as in Figure 1.

DISCUSSION

Rapid cardiac pacing produces early LV dilation, which increases progressively with the duration of pacing along with the decline of LV mechanical function (11). Left ventricular systolic dysfunction was evidenced in this study by an increase in LV end-diastolic pressure, and decreases in dP/dt and FS, although direct measurement of LV contractility is lacking. Furthermore, like human cardiomyopathy, the animals with pacing cardiomyopathy showed increased plasma NE, beta-receptor downregulation, reduced sympathetic neuronal reuptake of NE and sympathetic nerve terminal abnormalities. This study extends our earlier observation of the protective effects of antioxidant vitamins on sympathetic nerve terminal function (8) to CHF.

Oxidative stress in CHF. The results of this study indicate that oxidative stress is present in the failing heart. Although our study does not identify the source of oxidative stress, oxygen free radicals may be generated from oxidized derivatives of NE (19), ischemic metabolic distress or activated cytokines such as tumor necrosis factor alpha (20). Our findings are consistent with the increase of myocardial thiobarbituric acid reactive substance and immunohistochemical visualization of lipid peroxides in cardiomyocytes after four weeks of rapid ventricular pacing in dogs (12). Increased mitochondrial production of superoxide also has been demonstrated directly using electron spin resonance spectroscopy in pacing-induced cardiomyopathy (12). Additionally, a progressive increase in myocardial lipid peroxidation has been shown to correlate with the severity of heart failure produced by myocardial infarction (MI) (21).

Antioxidant effects of vitamins in CHF. Effective inhibition of free radical production was produced by antioxidant vitamins in our study. Since these agents also attenuated the cardiac dysfunction and sympathetic nerve terminal abnormalities, we speculate that the beneficial actions of the vitamins on the heart muscle and sympathetic nerve endings are mediated, at least in part, via their antioxidant effects.

In this study, we chose the three commonly used antioxidant vitamins, not only because of their efficacy as antioxidants but also because of their clinical relevance and minimal toxicity (22). The doses of vitamins chosen for the study were 3 to 10 times the current recommended dietary allowances for humans and are within the human therapeu-

tic ranges (23). A similar vitamin regimen raised myocardial alpha-tocopherol levels 70% in animals (unpublished data). Alpha-tocopherol is a potent lipophilic antioxidant, located mainly at the membrane surface (24,25). Beta-carotene is more lipophilic than alpha-tocopherol and can penetrate into the cell membrane deeper than alpha-tocopherol. Thus, beta-carotene is effective in quenching singlet oxygen in inner cells and complements the action of alpha-tocopherol, which acts primarily in the cytoplasmic membrane (26). On the other hand, ascorbic acid is the most important antioxidant in extracellular fluids (27). It traps peroxyl radicals in the aqueous phase and inhibits lipid peroxidation. Ascorbic acid reacts with tocopheroxyl radicals to yield tocopherol and an ascorbic radical at the surface of the cell membrane, thus regenerating reduced tocopherol and transferring the oxidative challenge to the aqueous phase (28). These synergistic effects of ascorbic acid, alpha-tocopherol and beta-carotene may help explain the greater antioxidant effects of the vitamin mixture compared with that produced by alpha-tocopherol alone. Furthermore, the fact that alpha-tocopherol produced greater inhibition of mtDNA 8-oxo-dG than cellular glutathione oxidation in the alpha-tocopherol alone group is consistent with the lipophilic property of alpha-tocopherol and suggests the need to include a hydrophilic antioxidant in an effective antioxidant treatment regimen. However, despite near complete abolition of the oxidative stress as judged by the changes in the tissue GSH/GSSG ratio and mtDNA 8-oxo-dG in the animals with CHF treated with vitamins, LV mechanical function remained depressed. These findings suggest that other factors are operative in the pathogenesis of cardiac depression in pacing-induced cardiomyopathy. Furthermore, despite the similarities in cardiac hemodynamics, neurohormonal profiles and oxidative stress between the pacing-induced cardiomyopathy and human cardiomyopathy, results of this study should be extrapolated with caution to clinical CHF in which the heart is not artificially paced at fast rates.

Clinical implications. The information on the effects of antioxidant vitamins in human CHF is scarce. However, since a significant correlation exists between the oxidative stress and the severity of CHF (2,29,30), one may assume that antioxidant therapy is beneficial. A study of patients with CHF showed that vitamin E reduces plasma levels of malondialdehyde and superoxide anion and elevates the levels of antioxidant enzymes (3).

Combined treatment with vitamins C and E suppressed neutrophil-mediated free radical production and lowered blood lipid peroxidation product in patients with acute MI (31). Oral administration of vitamins A and E for five days before surgery also reduced the oxidative stress after reperfusion with coronary artery bypass grafting (32). Other studies have shown that vitamin C improves endothelial function of conduit arteries (33) and prevents nitrate tolerance in patients with CHF (34). It also augments the inotropic response to dobutamine in human subjects with normal LV ejection fraction (35). Vitamin E reduces coronary atherosclerosis progression using serial angiography (36). The combination of vitamins A, C and E reduces infarct size and cardiac events during the first 28 days after onset of acute MI (37). Collectively, the studies indicate that the oxidative stress levels contribute to the adrenergic regulation of vascular tone and ventricular performance. Administration of antioxidant vitamins may affect the resting vasomotor tones and adrenergic responses of the blood vessel and the heart. However, none of the clinical studies include assessment of cardiac function. The long-term effects of vitamin E on cardiovascular events have been studied, but the results are conflicting. In the Cambridge Heart Antioxidant study (38), vitamin E reduced the incidence of nonfatal events in patients with coronary disease over a median follow-up period of 17 months, but there was a trend to increased mortality. In the Heart Outcome Prevention Evaluation study, vitamin E administered over a longer period exerted no beneficial cardiovascular effects in high-risk subjects, including patients with coronary artery disease with and without LV dysfunction (39). Vitamin E supplement also produced no significant beneficial effects in patient after MI (40). The reasons for the discrepant results are not known but may relate to the different patient characteristics and the use of concomitant medications (41). The role of vitamin E, either alone or in combination with other antioxidants, in human CHF deserves further investigation.

Acknowledgments

The authors thank Miss Janice Gerloff and Mrs. Robin Stuart Buttles, BS, for their excellent technical support.

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