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Luc Rochette [a], Etienne Tatou[b], Véronique Maupoil[a], Marianne Zeller[a], Yves Cottin[ab], Sahed Jazayeri[b], Roger Brenot[b], Claude Girard[ab], Michel David[b], Catherine Vergely[a]*

[a] LPPCE, Facultés de Médecine et de Pharmacie, IFR Santé-STIC, Dijon, France

[b]Centre Hospitalier Universitaire, Dijon, France

*Correspondence to: Professor Catherine VERGELY

Laboratoire de Physiologie et Pharmacologie Cardiovasculaires Expérimentales (LPPCE)

Facultés de Médecine et de Pharmacie, 7 Bd Jeanne d'Arc, BP 87900

21079 DIJON Cedex, FRANCE

Phone: (+33) 380 393 460 Fax: (+33) 380 393 293

Email: cvergely@u-bourgogne.fr

Running title: Oxidative stress in HF patients

Authors' contribution:
Designed research: LR, VM, YC, RB, MD
Performed study: ET, MZ, SJ, CG, CV
Analysed data, wrote paper: LR, CV

Funding sources: French Minister of Research, Conseil Régional de Bourgogne

Declaration of interest statement: The authors have no personal, financial or other relationships with other people or organizations within 3 years of beginning the work submitted that could inappropriately influence, or be perceived to influence, the work submitted.

ABSTRACT

Heart failure is characterized by a great number of metabolic and histological defects, however, previous studies did not provide strong evidence of a correlation between the antioxidant status of myocardial tissue itself and cardiac function. The goal of our study was to assess, in patients with heart failure consecutive to dilated cardiomyopathy (DCM), alterations in norepinephrine (NE), lipid peroxidation (malonedialdehyde: MDA) and iron levels in different parts of the myocardium and aorta, in relation to functional parameters.

Biopsied heart samples were obtained from 12 DCM patients and from 4 brain-dead organ donors (Controls). The left ventricular ejection fraction (LVEF) was reduced to 19.1±2.6% in DCM. For all patients, the distribution of NE in the atria, ventricles and vessels was different, but NE content in control hearts was systematically higher than in cardiomyopathy patients. MDA levels tended to be higher in the different samples from the DCM group in comparison with the values obtained in the C group; the values were significantly decreased ($p<0.05$) in endocardium and the aortic samples. In the right atrium there was a significant correlation between NE content and LVEF and between MDA and iron concentrations.

These findings could give further insights into the relationship between iron metabolism disturbances and the severity of cardiovascular diseases.

Keywords: norepinephrine, lipid peroxidation, iron, heart failure, patients

List of abbreviations:

CCPPRB: committee for the protection of people in biomedical research

CHU: regional hospital centre

CI: cardiac index

DCM: dilated cardiomyopathy

EDTA: ethylene diamine tetracetate

GPx: glutathione peroxidase

HPLC: high-performance liquid chromatography

LVEF: left ventricular ejection fraction

MDA: malonedialdehyde

MMP: matrix metalloproteases

NE: norepinephrine

OS: oxidative stress

PCWP: pulmonary capillary wedge pressure

SOD: superoxide dismutase

TBA: thiobarbituric acid

TBARs: thiobarbituric acid reactive substances

TCA: trichloroacetic acid

Introduction

A number of mechanisms have been put forward to explain the pathogenesis of cardiomyopathies associated with clinical manifestations. It is likely that the cause is multifactorial. Previous studies indicate that terminal stage heart failure is characterized by extensive myocardial damage with altered structure of the myocardium [1]. One important compensatory mechanism that maintains adequate tissue perfusion during functional impairment of the heart is the increase in the failing cardiac output by a compensatory increase in the activity of the sympathetic nervous system. Many years ago, it was shown that myocardial levels of norepinephrine (NE) were depleted in patients with congestive heart failure, even though NE turnover was increased [2]. Marked regional variations in levels of NE have been reported in cardiomyopathic left and right ventricles [3, 4] and, on the other hand, experimental studies have shown that catecholamines were able to induce oxidative stress (OS) in the heart [5]. It appears then that the relationships between NE metabolism and changes in oxidative stress are not clearly demonstrated.

Dysfunction in the failing myocardium may result from oxidative stress disequilibrium [6, 7]. Growing evidence suggests that oxidative stress modification may be induced by ionic metals such as iron and cupper. Iron is an essential trace element that contributes to the destabilization of redox potential, in particular with regard to the hydroxyl radical produced in Fenton‘s reaction [8]. Under normal physiological conditions, body iron is well protected from taking part in uncontrolled oxidative reactions [9, 10]. It has been suggested that modifications in its metabolism was able to contribute to coronary heart disease [11, 12]. Studies have presented evidence that heart failure situations are characterized by a great number of metabolic and histological defects that occur during different stages of the disease [13-15]. However, in these studies the results did not provide strong evidence of a correlation

between the antioxidant status of myocardial tissue and cardiac function; the biological parameters were evaluated in the patients' plasma but not in their tissue.

Thus, the purpose of the present study was to investigate both levels of NE, iron and the lipid peroxidation index in different parts of explanted hearts and aortic tissue obtained from patients with dilated cardiomyopathy (DCM). The relationships between NE signalling, oxidative stress and functional parameters were evaluated.

Materials and Methods

Ethics statement

The study was conducted in accordance with the Helsinki Declaration of 1975 (revised 1983) and institutional ethical regulations. Explanted hearts were obtained from patients undergoing cardiac transplantation for ischemic cardiomyopathy. These patients suffered from heart failure clinically classified according to the New York Heart Association class IV CHF on the basis of clinical symptoms and signs as assessed by the cardiologist shortly before the operation; all patients gave written informed consent to participate in the study. For the normal control subjects (non-failing hearts rejected for transplantation for clinical reasons, suspicion of infection and liver disease), written family consent was provided on behalf of deceased organ donors. The study design was approved by the Ethics Committee for Organ Transplant and Local Committee for the Protection of People in Biomedical Research (CCPPRB, CHU Bocage, Dijon, France) before the study began.

Patients and myocardial samples

Myocardial and vascular tissues from the heart were obtained from 12 consenting patients with DCM who underwent orthotopic cardiac transplantation. Cardiac medication of these patients included angiotensin-converting enzyme inhibitors, angiotensin-2 type-1 receptor antagonists, beta-blockers, diuretics and aldosterone antagonists. Hearts from brain-dead organ donors (n=4) that were rejected for transplantation for clinical reasons were included in this study (Controls). Regarding the donor-related characteristics, dopamine (10µg/kg/min) and dobutamine (10 µg/kg/min) had been administered to the 4 donors during intensive care. The waiting period between the diagnostic of brain-dead and the start of the cross-clamping period has been to 6-8h. No more detail on the indication of treatment during intensive care before organ donation had been obtained from the hospitals.

The hearts from patients with DCM were arrested in situ with ice-cold St Thomas' Hospital cardioplegic solution. The explanted heart was placed on ice immediately after removal from the body and transported to the laboratory within 20 min in ice-cold cardioplegic solution. Selected clinical features of the transplant recipients are shown in Table 1.

The hearts were sectioned for analysis. In each one, the left ventricle (endocardium and epicardium), left and right atrium and samples of the initial part of ascending aorta were also excised. Frozen specimens were crushed under liquid nitrogen to obtain a powder. In order to reduce the effect of variations between specimens, the levels of NE and lipid peroxidation were expressed as nM/g protein. Protein levels were measured according to the Lowry method.

Measurement of norepinephrine

Tissue NE was assayed by high-pressure liquid chromatography with electrochemical detection [16]. Each fragment was homogenized on ice for 1 minute in a solution containing

0.2 M perchloric acid, 2.7 mM ethylene diamine tetracetate (EDTA), 5.26 mM sodium metabisulfite, and 100 µl of an internal standard, 3,4-dihydroxybenzylamine hydrobromide. The samples were centrifuged at 13,000g for 6 minutes at 4°C. The supernatants were analyzed by high-performance liquid chromatography (HPLC) with a reverse phase column (60 RP Select B, 250 x 4 mm, 5 µm), with electrochemical detection. The potential of the electrochemical detector was set at +0.85V. The mobile phase consisted of 0.08 M sodium phosphate, 0.27 mM EDTA, 3.7 mM octan-1-sulfonic acid at pH 3.5.

Measurement of lipid peroxidation

The hearts were homogenized in 5 volumes of 50 mM phosphate buffer at pH 7.4 containing 0.1 mM EDTA or in 5 volumes of a 0.25 M sucrose solution. Lipid peroxidation (malondialdehyde: MDA) in tissues was evaluated by the thiobarbituric acid reaction (TBAR); using spectrofluorimetry [17] . Cardiac homogenates were combined with the trichloracetic acid (TCA)-TBA-hydrochloric acid (13.5% w/v, 0.33% w/v, 0.85 *N* respectively) reagent and thoroughly mixed. The tubes were boiled for 15 min and cooled in a bucket of ice. TCA (70%) was then added and after 20 min of incubation, the flocculent precipitate was removed by centrifugation. The fluorescence was measured at 515 nm excitation and 553 nm emission. Thiobarbituric acid reactive substances (TBARS) were expressed as nM of MDA per gram of proteins.

Measurement of iron in tissues

The tissues were wet ashed in nitric acid using a Kjeldhal apparatus. The samples were digested for 4 h and diluted with deionized water. Using appropriate standards to establish linear standard curves, iron concentrations in tissues were determined by reaction with ortho-

phenanthroline to form a coloured complex [10]. The most strongly absorbed light is found by measuring the absorbance between 400-600 nm. The values were expressed as µg/g protein.

Statistical analysis

Comparisons between control tissue and tissue from pathological hearts were made using the Student's t-test or the Mann-Whitney test. A probability of ≤ 0.05 was considered significant. All data are reported as the mean ± S.E.M.

Results

Hemodynamic parameters

The hemodynamic parameters in each group are given in Table 1. At cardiac catheterization, left ventricular dysfunction was present at rest with a mean pulmonary capillary wedge pressure (PCWP) of 26.3 ± 2.7 mmHg in the group of patients with cardiomyopathy. The preoperative left ventricular ejection fraction (LVEF) was low in all patients; the mean LVEF was 19.1 ± 2.6 %; (10 patients in end-stage heart failure: LVEF values < 22 %.). The LVEF values in control hearts were between 50 and 60%.

Norepinephrine levels

The data for NE content in the different parts of the hearts and vessels are summarized in Table 2 .There were significant differences in the concentration of NE in the various parts of the myocardium. There were several differences in the distribution of NE among the various subdivisions of the atria and ventricles studied. NE content was consistently and significantly higher in the right atria than in the left atria or in the ventricles (right atria and left atria, respectively: 6.78 ± 3.25 ng/mg prot and 3.32 ± 1.21 ng/mg prot in the cardiopathy group and

19.10 ± 0.89 ng/mg prot and 6.95 ± 0.26 ng/mg prot in the C group). The hearts of DCM patients had lower levels of NE in the left ventricle than did control hearts ($p<0.05$). NE levels in the aorta were very low in comparison with levels in the different parts of the myocardium. A significant correlation between NE levels and LVEF (%) ($p = 0.022$) was observed in the samples of left atria from patients (Fig 1).

MDA levels

Tissue levels of MDA for the regions of the heart and vascular samples are given in Table 3. There was considerable variation in MDA concentrations between the hearts of the patients with cardiomyopathy and controls. MDA levels tended to be higher in the different samples from the DCM group in comparison with the values obtained in the C group; the values were significantly decreased ($p<0.05$) in endocardium and the aortic samples. Levels of iron in the myocardium and aorta did not differ significantly between hearts from the cardiomyopathy patients and control hearts.

Iron levels

A significant correlation between MDA and iron concentrations in the right atrium was found in groups of patients with DCM $p=0.008$ (Fig 2). In contrast, no significant correlation was noted in the left atrium. We found no correlation between levels of MDA and iron in other parts of the myocardium or vessels from the same patients.

Discussion

Levels of NE in tissue showed a regional variation that was apparent in all hearts (controls or DCM group). We observed a higher NE concentration in the right atrium than in the left. NE levels in the aorta were low in comparison with those found in the ventricles or atria. Our study is in accordance with previous results showing that NE concentrations in the different parts of the heart may vary significantly [18, 19]. Heterogeneous regional function determined by magnetic resonance imaging has been described in patients with hypertrophic cardiomyopathy [20]. This heterogeneity may reflect regional variations in myocardial dysfunction and fibrosis that is characteristic of this pathology. Heng et al. [21] suggested the importance of geometrical determinants of altered intramyocardial strain in some areas of the myocardium. It has been reported that hypertrophic cardiomyopathy involves sympathetic dysinnervation [22] and that dilated cardiomyopathy is frequently associated with regional neuronal dysfunction [23]. This may reflect variations in wall stress, but also the regional and heterogeneous loss of neuronal tissue. In our study, the right ventricles of cardiomyopathy patients appeared to have depleted levels of NE [4]. Previous results showed a similar phenomenon in both ventricles for a variety of biochemical changes in heart failure [27-29].

An important result of our work lies in the fact that we showed for the first time a correlation between the values of preoperative LVEF and NE concentrations in the left atria of patients with cardiomyopathy. This result may reflect variations in wall stress in the left atrium but also a mechanism of chronic adaptation. It has been reported that several neurohormonal factors, including catecholamines are involved in the process of cardiac remodelling associated with heart failure [24].

The development and progression of heart failure depends on a variety of cellular and molecular abnormalities including oxidative stress, mediated by the generation of oxygen free

radicals [5,6]. The present study demonstrates that in failing human myocardium, the endocardium of the left ventricle and aorta showed higher levels of lipid peroxides compared with controls ($p < 0.05$). Our results are in agreement with others that showed an increase in myocardial MDA content in the failing human myocardium [25]. In the study by Maack et al [25], lipid peroxidation was measured in only one part of the ventricular myocardium.

An important question addressed in our study is the difference between the results for the two atria. The NE content was consistently and significantly higher in the right atria than in the left atria or in the ventricles. It is important to observe that the atria of DCM patients had lower levels of NE that did control atria. MDA levels tended to be higher in the atria of the DCM group in comparison with the values obtained in the C group. A significant correlation between MDA and iron concentrations in the right atrium was observed in groups of patients with DCM. It is well documented that iron overload causes oxidative damage, and cardiomyopathy [26, 27]. To our knowledge, this is the first demonstration of a decrease in NE associated with an increase in the oxidative index; a significant correlation between this index and iron content was observed in the right atria of patients with heart failure. These results reinforce the concept that chronic heart failure is characterised in the atria by excessive adrenergic activity and an increase in oxidative stress. A recent report indicates that levels of endogenous antioxidant agents such as glutathione are reduced in left atrial samples from patients with chronic heart failure, suggesting that abnormal oxidative stress balance plays a role in human heart failure [28, 29]. Oxidative stress has also been implicated in forms of atrial remodelling [30].

Concerning our results for the aortic samples, it appears that the levels of NE in the aorta were very low in comparison with the levels in the different parts of the myocardium. Only a few studies in human vessels have been performed to date. In general, the distribution of NE in different regions of the heart and vessels has been examined using experimental approaches.

The vessels contain lower concentrations of NE than do the ventricles or atria. These findings are in agreement with the distribution of adrenergic receptors and the expression of adenylcyclase subtypes associated with these receptors [31]. Our results are in accordance with the recent results reported by Jensen et al [32]. These authors found that, in humans, the absolute level of alpha-adrenergic receptors was lower in the arteries than in the left ventricle.

One limitation of our study relates to the control group; the control and heart failure groups were not matched for age and medications. However, it is not easy to obtain cardiac tissue biopsies from patients without heart failure. Furthermore, it is obvious that the cardiac medications may be able to modulate adrenergic stress and oxidative status in heart-failure patients and brain-dead organ donors; the control patients receiving dopamine and dobutamine. These are unavoidable limitations of such studies using human myocardial samples.

In summary, we demonstrated that significantly lower levels of myocardial and vascular NE were associated with an increase in peroxidation in patients with heart failure secondary to dilated cardiomyopathy. Our study confirms the hypothesis that oxidative stress plays a role in human heart failure. We have demonstrated a significant correlation between lipid peroxidation and iron content in the right atrium; these finding could provide further insights into the relationship between disturbances in iron metabolism and the severity of cardiovascular diseases, such as atrial fibrillation. A significant correlation between MDA and iron concentration was observed in the right atrium suggesting that in some cardiovascular pathologies such as heart failure, the iron status is modified.

Acknowledgments

We would like to thank Claude Mossiat for the technical assistance and Philip Bastable for English proof reading.

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Table 1: Clinical characteristics of heart transplant recipients

	Dilated cardiomyopathy (DCM)	**Controls (C)**
Sex M/F	10/2	4/0
Age (Yr)	54 ± 2	18, 25 27, 37
Left ventricular ejection fraction (%)	19.1 ± 2.6	50,50, 55, 60
Cardiac Index (litres/min per m^2)	1.9 ± 0.1	ND
Pulmonary capillary Wedge pressure (mmHg)	26.3 ± 2.7	ND

Means ± SEM
ND: not determined

Table 2: Levels of norepinephrine (NE) in different parts of the myocardium

	Dilated cardiomyopathy (DCM)		**Controls (C)**	
Left ventricle		n		n
Endocardium	2.99 ± 0.32 a	12	7.23 ± 0.36	4
Epicardium	4.74 ± 0.74 a	12	9.33 ± 0.34	4
Atria				
Left	3.32 ± 1.21 a c	12	6.95 ± 0.26 c	4
Right	6.78 ± 3.25 b	12	19.10 ± 0.89	4
Initial part of ascending aorta	1.16 ± 0.15 a	12	1.95 ± 0.075	4

Norepinephrine (NE) levels: ng/mg protein
Means ± SEM
a $p<0.05$ C vs. DCM
b $p<0.01$ C vs. DCM
c $p<0.05$ left atrium vs. right atrium

Table 3: Tissue levels of malonedialdehyde (MDA) and iron in different parts of the myocardium

	Dilated cardiomyopathy (DCM)				Controls (C)			
	Malonedialdehyde		**Iron**		**Malonedialdehyde**		**Iron**	
Left ventricle		n		n		n		n
Endocardium	152 ± 38 a	12	51 ± 6	12	74 ± 6	4	56 ± 4	4
Epicardium	121 ± 19	12	58 ± 6	12	64 ± 3	4	63 ± 5	4
Atria								
Left	129 ± 36	11	45 ± 7	11	58 ± 5	4	57 ± 6	4
Right	132 ± 35	12	48 ± 7	12	93 ± 2	4	59 ± 7	4
Initial part of ascending aorta	193 ± 54 a	12	51 ± 8	12	30 ± 1	4	61 ± 4	4

Malonedialdehyde levels: nM/g protein
Iron levels: µg/g protein
Means ± SEM

a $p<0.05$ C vs. DCM

Figure Legends:

Figure 1: Correlation between NE levels in the left atrium of the heart (ng/mg prot) and LVEF (%) (DCM patients, n=12) $r = +0.642$, $p = 0.022$

Figure 2: Correlation between iron levels (µg/g protein) and MDA values in (nM/g protein) in the right atrium of the heart (DCM patients, n=12) $r = +0.723$, $p = 0.008$

Figure 1:

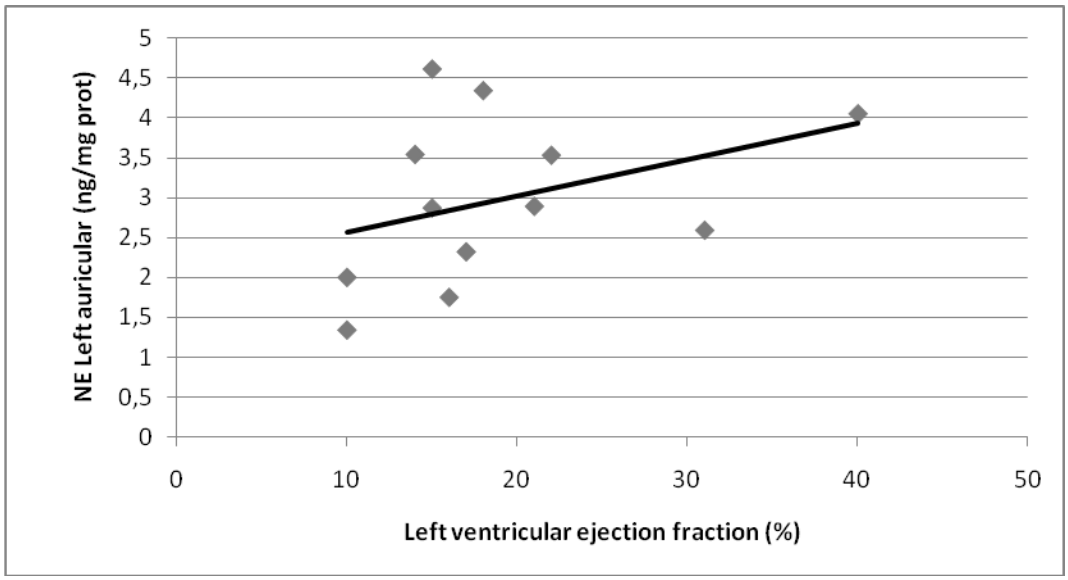
NE Left auricular (ng/mg prot)
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
0
10
20
30
40
50
Left ventricular ejection fraction (%)

Figure 2