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Chronic treatment with Carvedilol improves ventricular function and reduces myocyte apoptosis in an animal model of heart failure

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Abstract

Background: β-blocker treatment has emerged as an effective treatment modality for heart failure. Interestingly, β-blockers can activate both pro-apoptotic and anti-apoptotic pathways. Nevertheless, the mechanism for improved cardiac function seen with β-blocker treatment remains largely unknown. Carvedilol is a non-selective β-blocker with α-receptor blockade and antioxidant properties. We therefore studied the impact of the effects of carvedilol in an animal model of end-stage heart failure.

Results: To test whether chronic treatment with β-blockade decreases apoptosis, we treated myopathic turkeys with two dosages of carvedilol, 1 mg/kg (DCM_1) and 20 mg/kg (DCM_{20}), for four weeks and compared them to non-treated DCM animals (DCM_0) and to control turkeys (CON). Echocardiographic measurements showed that non-treated DCM animals had a significantly lower fractional shortening (FS) when compared to CON (68.73 ± 1.37 vs. $18.76 \pm 0.59\%$, $p < 0.001$). Both doses of carvedilol significantly improved FS (33.83 ± 10.11 and $27.73 \pm 6.18\%$ vs. 18.76 ± 0.59 % for untreated DCM, $p < 0.001$). DCM left ventricles were characterized by a higher percentage of apoptotic nuclei when compared to CON (5.64 ± 0.49 vs. $1.72 \pm 0.12\%$, respectively $p < 0.001$). Both doses of carvedilol significantly reduced the number of apoptotic nuclei ($2.32 \pm 0.23\%$ and $2.36 \pm 0.26\%$ 1 mg and 20 mg/kg respectively).

Conclusions: Carvedilol improves ventricular function. Furthermore, treatment with carvedilol decreased the incidence of apoptosis in cardiac myocytes from failing hearts at both doses. These data suggest that the inhibition of apoptosis with carvedilol may lead to improvement in ventricular function and may underlie a beneficial effect of β-blockade independent of heart rate lowering effects.

Background

Loss of myocytes is thought to contribute to the progressive decline in left ventricular function and the development of congestive heart failure. Recent studies have proposed that myocyte loss in cardiomyopathy can occur by apoptosis without an inflammatory response [reviewed in [1–3]]. In heart failure, growth stimulation initially occurs as a compensatory effort to meet chronically altered hemodynamic demands and is mediated by systemic and/or local up-regulation of mediators of adrenergic pathways and by various cytokines [4,5]. However, cytokines can be directly toxic to cardiac myocytes and result in increased apoptotic cell death [3,6]. Local up-regulation of angiotensin II induces immediate-early genes, which may lead to increased protein synthesis and myocardial hypertrophy or, alternatively, may up-regulate expression of apoptotic proteins (such as p53) in cardiac myocytes. The initial increase in circulating norepinephrine is thought to maintain cardiac function through inotropic mechanisms. However, a direct cardiac myocyte toxicity from norepinephrine is well recognized [6,7]. Furthermore, pressure-overload and stretch associated release of angiotensin II has been shown to induce myocyte apoptosis [8–10]. In an elegant paper by Telger et al., it has clearly been demonstrated that cardiac hypertrophy is initially preceded by a wave of apoptosis of cardiac myocytes followed by cell growth and a decrease in programmed cell death [10]. Apoptosis may, therefore, be the consequence of prolonged growth stimulation (adrenergic and renin-angiotensin axes) of adult cardiac myocytes, which are terminally differentiated. Similarly, certain cytokines (such as tumor necrosis factor) can induce growth as well as apoptosis [11]. As proposed by Telger et al. it is becoming clear that cell growth and programmed cell death are in fact two linked processes [10].

Over the last five years, β-blocker therapy has become one of the main treatment modalities for heart failure. Of particular interest is the use of carvedilol [12–16]. Carvedilol is a novel multiple-action neurohormonal antagonist. Its primary activities are nonselective β-adrenoceptor blockade, vasodilatation (mediated through α1-adrenoceptor blockade), and antioxidant activity [12]. In many clinical studies, carvedilol has been shown to improve left ventricular function and symptoms in patients with ischemic heart disease or idiopathic dilated cardiomyopathy [13–16] and to dramatically reduce mortality [17]. However, it remains unclear how carvedilol improves ventricular function in heart failure.

In an animal model of cardiomyopathy that has many similarities to human heart failure, we tested the hypothesis that a reduction in apoptosis may be responsible for the beneficial effects of carvedilol. As such, attenuation of apoptosis in the myocardium may represent a novel and important mechanism that contributes beneficial effects on the myocardium.

Results

Baseline Characteristics of Dilated Cardiomyopathic Animals

We have previously shown that removal of furazolidone from the feed after three weeks results in progressive cardiac dilatation and heart failure [18]. At the time of randomization of the DCM group into three groups i.e., DCM_0 (no treatment), DCM_1 (lower dose carvedilol) or DCM_{20} (higher dose carvedilol), fractional shortening was not different between the groups ($p > 0.1$). Evidence for severe heart failure was marked by significantly reduced left ventricle fractional shortening (controls $63.67 \pm 1.04\%$ n = 34 vs. DCM $11.95 \pm 0.59\%$ n = 18, $p < 0.001$).

Effect of Carvedilol on Ventricular Function

We investigated the effect of carvedilol by administering two different dosages (1 mg and 20 mg/kg body weight per day) to control and DCM turkeys for four weeks. We studied two dosages (pharmacological and non-pharmacological) as a difference in dose-dependency for cardiac function improvement has previously been demonstrated by us with another non-selective beta-blocker [18]. Furthermore, we wanted to determine if carvedilol might exert beneficial effects independent of heart rate effects. The lower dose (1 mg/kg) did not reduce heart rate. However, the higher dose reduced HR by approximately 15% for 8 hours.

In vivo contractile performance of the heart was assessed by the determination of fractional shortening of the left ventricle. As shown in Table 1 and Figure 1, fractional shortening was increased by 32 and 45% in DCM_1 and DCM_{20} animals, respectively, compared to non-treated DCM animals (DCM_0). Compared with baseline values at the time of randomization, the improvement in fractional shortening was of 57% in DCM_1 and 65% in DCM_{20}. Associated with improved cardiac function was a reduction in the incidence of apoptotic nuclei (see below).

The body weight was significantly decreased in DCM animals compared to control animals ($p < 0.001$ compared to control) (Table 2). This animal model is associated with cachexia. Carvedilol treatment resulted in a slight gain of body weight, 15% in the DCM_1 group and 20% in the DCM_{20} group compared with the non-treated DCM group (DCM_0). Carvedilo also increased the body weight of control animals ($p < 0.001$). These data indicate a favorable effect on cachexia. In non-treated DCM animals the hearts weighed significantly less than control hearts ($p = 0.01$). DCM animals treated with carvedilol showed a significant increase in HW compared to hearts from non-treated DCM animals ($p < 0.001$). Therefore the HW/BW

Table 1: Inhibition of Apoptosis by Carvedilol Correlates with Improvement in LV Function.

	Cont n = 13	Cont$_1$ n = 11	Cont$_{20}$ n = 10	DCM$_0$ n = 18	DCM$_1$ n = 13	DCM$_{20}$ n = 12
FS %	68.73* ± 1.37	66.09* ± 0.90	66.99* ± 1.37	18.76 ± 1.83	27.73* ± 6.18	33.83* ± 10.11
Apoptosis %	1.72* ± 0.12	2.10 * ± 0.25	2.20* ± 0.24	5.64 ± 0.49	2.32* ± 0.23	2.36* ± 0.26

Fractional shortening (FS%) was calculated from echocardiographic images as described in the methods. Apoptotic nuclei were counted as described in methods. *$p < 0.001$ compared to DCM$_0$.
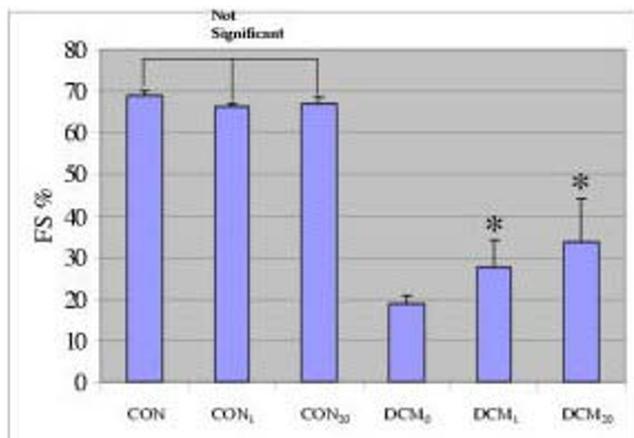
Figure 1
Effect of carvedilol on fractional shortening (FS) of the left ventricle. Carvedilol administration is associated with a improvement in left ventricular function DCM$_0$ (n = 18), DCM$_1$ (n = 13), DCM$_{20}$ (n = 12). *$p < 0.001$ compared to DCM$_0$. Healthy control turkeys (CON); control turkeys treated with two dosages of carvedilol, 1 mg/kg (CON$_1$) and 20 mg/kg (CON$_{20}$); dilated cardiomyopathic turkeys (DCM); non-treated DCM animals (DCM$_0$); myopathic turkeys treated with two dosages of carvedilol, 1 mg/kg (DCM$_1$) and 20 mg/kg (DCM$_{20}$).

ratio was lower for hearts from carvedilol treated DCM animals compared to hearts from non-treated DCM animals. Interestingly, the HW/BW ratio was lower for carvedilol treated control animals compared to non-treated control animals. Left ventricle volume was significantly larger for non-treated DCM hearts. Carvedilol significantly decreased LV volume at both dosages.

Effect of Carvedilol on Cardiac Myocyte Apoptosis
Evaluation of tissue sections by the TUNEL technique revealed only small numbers of TUNEL-positive cells in the control hearts (Table 1 and Figure 2A). Hearts from DCM animals had a significant increase in the number of TUNEL-positive cells compared to controls ($5.64 ± 0.49\%$ vs. $1.72 ± 0.12\%$ DCM and control respectively, p <

0.001), indicating the occurrence of apoptosis (Figure 2B).

In contrast to non-treated DCM hearts, myocardial tissue sections prepared from hearts from animals treated with carvedilol showed a significant reduction in the numbers of TUNEL-positive myocyte nuclei. An example of such an observation is depicted in Figure 3A and 3B (DCM$_1$ and DCM$_{20}$ respectively). Both the lower and the higher doses of carvedilol were effective in reducing apoptosis from $5.64 ± 0.49\%$ in non-treated DCM animals to almost control levels $2.32 ± 0.23\%$ (DCM$_1$) and $2.36 ± 0.26\%$ (DCM$_{20}$), $p < 0.001$, compared to DCM$_0$ respectively (Figure 3C).

Discussion
Effect of Carvedilol on a Heart Failure Model
The furazolidone-induced turkey model is a well characterized experimental model of dilated cardiomyopathy with features very similar to those found in human heart failure [19–22]. Decreased contractile function (reduced fractional shortening of the left ventricle) and morphological changes (increased heart volumes, left ventricular dilatation, and myocyte hypertrophy) are characteristic features of this model [21,22].

β-blocker treatment has emerged as an effective treatment modality for heart failure. Both in animal and human studies of heart failure, β-adrenergic blockade intervention appears to have beneficial effects on cardiac myocyte function [1,23,24]. Of particular importance, studies using carvedilol have shown remarkable improvements in cardiac performance [12,13,15,16] and reduction in mortality in humans [17]. We have previously shown that propranolol, a nonselective β-blocker, is cardioprotective and prevents the development of heart failure in turkeys when given concurrently with furazolidone [21]. Similarly, carteolol, a nonselective β-blocker, resulted in improvement in ejection fraction, reduction in ventricular volumes, an increase in developed pressure as well as an increase in rate of survival in our turkey model [18]. Improvement in cardiac function was associated with cellular remodeling of cardiac myocytes. Cardiac myocytes from hearts treated with a non-selective β-blocker i.e.,

Table 2: Effect of Carvedilol on Gross Morphology

	HW (kg)	BW (kg)	HW/BW (%)	LV vol (ml)
Control (n = 13)	0.018✳ ± 0.001	4.36# ± 0.16	0.4★ ± 0.013	0.24• ± 0.04
$Cont_1$ (n = 11)	0.018✳ ± 0.001	5.3# ± 0.14	0.35★ ± 0.016	0.37• ± 0.06
$Cont_{20}$ (n = 10)	0.02✳ ± 0.001	5.65# ± 0.09	0.35★ ± 0.008	0.37• ± 0.04
DCM_0 (n = 18)	0.017 ± 0.001	2.61 ± 0.23	0.64 ± 0.049	9.36 ± 2.0
DCM_1 (n = 13)	0.019✳ ± 0.001	3.07# ± 0.29	0.60★ ± 0.05	6.28• ± 2.54
DCM_{20} (n = 12)	0.019✳ ± 0.001	3.26# ± 0.29	0.58★ ± 0.078	5.38• ± 1.95

HW: heart weight ✳ $p \leq 0.01$ compared to non-treated DCM group; BW: body weight #$p < 0.001$ compared to non-treated DCM group; HW/BW ratio ★ $p < 0.04$ compared to non-treated DCM group; LV vol: atria were removed and the left ventricles were arrested in diastole and filled with saline • $p \leq 0.008$ compared to non-treated DCM. Note the increase in body weight and heart weight with carvedilol treatment. This resulted in a significant improvement in HW/BW ratio despite having heavier hearts. The reduction in heart weight seen in the non-treated DCM group was associated with a higher incidence of apoptosis while the increased heart weight in hearts from DCM animals treated with carvedilol was associated with a lower incidence of apoptosis.

carteolol, had normal calcium cycling, normal myocardial energetics, normal β_1 receptor density as well as regression of myocyte hypertrophy and reduced connective tissue content [18]. Furthermore, administration of carteolol has been reported to prevent the development of virally induced cardiomyopathy in a murine model [25]. In a canine model with left ventricular dysfunction produced by multiple sequential intracoronary microembolizations, long term treatment with metoprolol, a β_1-selective blocker, has been reported to prevent the progression of LV systolic dysfunction and LV chamber dilatation [26]. Similarly, our present findings on the effect of carvedilol indicated improvement of left ventricular function and smaller LV volumes. Carvedilol prevented progression of LV systolic dysfunction and LV dilatation as previously reported in other models using β-blockers [18,26].

Animals with dilated cardiomyopathy which received carvedilol showed significant improvement in LV systolic function after 4 weeks. Notably, the improvement of LV systolic function in animals treated with carvedilol was associated with a decrease of LV volumes compared with non-treated DCM animals. Importantly, both doses of carvedilol improved LV contractile performance. Fractional shortening (%) was increased by an average of approximately 38% and impressively by 80% in some individual animals. These data seem to agree with the results of human clinical studies, and are suggestive that carvedilol not only slows deterioration of cardiac function, but also improves cardiac function.

The trend that the 20 mg/kg body weight dose might not have been well tolerated was demonstrated by some animals becoming moribund for a short period after dosing which might be due to a reduction in heart rate and car-

diac output with resultant reflect tachycardia. We have previously shown a negative force-interval relationship (i.e., negative treppe) in failing hearts with this model [18]. This is in line with data from human studies, which showed that when first administered, β-blockers actually slow heart rate and diminish ejection fraction [27]. The higher dose of carvedilol significantly reduced heart rate and blood pressure for up to eight hours. Animals that tolerated the higher dose did nevertheless benefit from the treatment. This is similar to human clinical studies where greater benefit is derived from higher dosages, if tolerated [28].

Apoptosis in Animal Models of Heart Failure
Heart failure is characterized by progressive deterioration of global left ventricular function over time. The mechanisms responsible for the worsening of cardiac function are not clear. Loss of cardiac myocytes has been suspected to be a feature of the cardiomyopathic process that contributes to progressive decline in left ventricular function and the development of congestive heart failure [29]. Evidence supporting the concept of myocyte apoptosis occurrence and contribution in the progression of heart failure has been obtained from a variety of observations, including *in vitro* studies, experimental animal models of cardiac dysfunction, and studies on cardiac tissue obtained from patients with end-stage heart failure [5,7–9,29].

We sought to evaluate the incidence and the extent of apoptosis in our animal model of heart failure. Using the TUNEL technique as a means of detection, we were able to document the occurrence of apoptosis in ventricles from failing hearts, which was characterized by a higher percentage of apoptotic nuclei when compared to controls (5.64% vs. 1.72%). These apoptotic indexes are within the
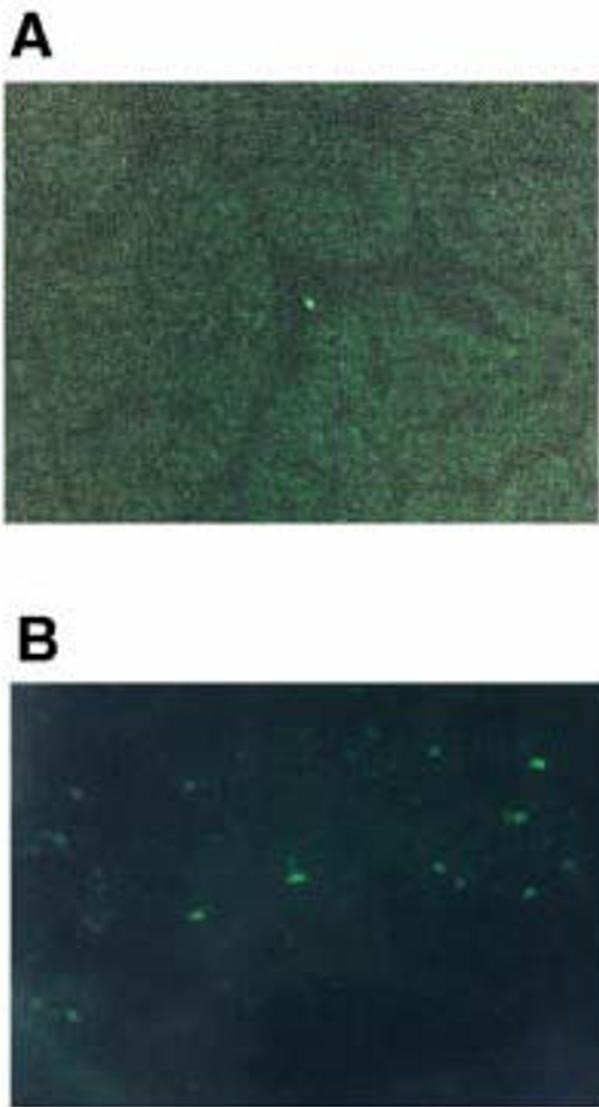
Figure 2
Detection of apoptotic cells by fluorescence microscopy. The TUNEL assay with fluorescein as the tag was used to stain the nuclei of apoptotic cells in frozen cardiac sections. (A) Control heart (containing essentially no TUNEL-positive cells in this field) and (B) a fluorescent micrograph of a representative field from a failing heart (DCM) containing a higher number of apoptotic nuclei. Micrographs were taken with 40X objective and reduced for reproduction.

Figure 3
Effect of carvedilol on cardiac myocyte apoptosis. Failing hearts treated with carvedilol show reduced number of TUNEL-positive nuclei, (A) DCM_1 and (B) DCM_{20}. Both low and high doses of the drug inhibited myocyte apoptosis to almost control levels (C). No effect of carvedilol on apoptosis in control groups was observed (C). *p < 0.001 vs. control and carvedilol treated groups. Micrographs in (A) and (B) were taken with 40X objective and reduced for reproduction.

range of values in heart failure reported in the literature (0.2% to 35%) [3,30–33]. The high degree of variability reported in the literature in the magnitude of apoptosis

may be due to the diversity and specificity of methods used to quantify apoptotic nuclei.

When reporting apoptotic nuclei using the TUNEL assay, two observations should be considered; 1) although apoptosis occurred in our model of heart failure, we might have missed, in random histological sections, evidence of apoptosis thereby under estimating the severity of programmed cell death due to the fact that apoptotic cells undergo rapid phagocytosis with the entire process lasting less than two hours in some cell systems [34], and 2) apoptosis in the present study was evaluated at a single time point when the heart was still undergoing LV remodeling. Hence, the documentation of apoptosis at this stage may not reflect the true magnitude of its occurrence during the initiation and transition to heart failure. In a rat model of pressure-overload hypertrophy produced by aortic banding, apoptosis appeared to peak at four days and gradually subsided after one month of aortic banding [9]. Therefore, a longitudinal study of apoptosis in failing hearts from initiation to transition to failure is worth pursuing. In this way, the incidence of apoptosis can be documented as the heart moves progressively from normal to compensated hypertrophy and finally to overt decompensated heart failure. These animals were in compensated heart failure and, often in the non-treated group, went into decompensated heart failure when stressed during echocardiographic examination.

With improved cardiac function, there should be a reduction in activation of the adrenergic-renin-angiotensin axes as well as a decrease in activated cytokines such as tumor-necrosis factor-α. In our model of heart failure, cardiac function correlated with the incidence of apoptosis. As described in Table 1 and Figure 3, there were a higher number of apoptotic cells in hearts with a reduction in fractional shortening. In contrast, control hearts with normal ventricular function showed low rates of apoptosis. The extent and occurrence of apoptosis may partially explain the lack of an overall increase in heart weight in hearts from non-treated DCM animals, despite a pronounced increase in heart size. These observations, and the results from several animal models, demonstrate that apoptotic cells are present in failing hearts, suggesting that apoptosis might be a mechanism involved, at least in part, in the progression of heart failure and the reduction of myocyte mass.

Carvedilol Inhibition of Apoptosis and Improvement of Ventricular Function

The extent of apoptosis in cardiomyopathic animals was significantly reduced by treatment with carvedilol. Both the lower and higher doses of carvedilol were effective in decreasing the rate of apoptosis (approximately a 58% reduction). This observation represents an important

finding in demonstrating the effectiveness of a cardiovascular drug to protect against continual cardiac myocyte apoptosis in failing hearts *in vivo*. Furthermore, the inhibition of apoptosis by carvedilol was accompanied by signs of improvement in left ventricular function and heart size (i.e. reduced LV volume) in the myopathic animals. Whether the improved function, small heart size and decreased wall stress were responsible for a reduction in apoptosis is not known. However, the derived cardiac benefit was independent of heart rate effects because benefit was obtained at both dosages.

Our data support the conclusions of Rossig et al. in endothelial cells. They reported levels of apoptotic cells for control and NYHA III-IV hearts that were similar to our observations [35]. We similarly believe that the suppression of apoptosis by carvedilol is likely due to its antioxidative properties rather than the β-blocking effects [35]. We base this on our observation that a similar reduction in the incidence of apoptosis was seen at both a non-pharmacological (no effect on heart rate or blood pressure) as well as a pharmacological dose of carvedilol. In a canine model of chronic heart failure produced by multiple sequential intracoronary embolizations, metprolol, a β_1 selective blocker, reduced the incidence of apoptosis supporting our experimental observations with carvedilol [36]. The incidence of apoptosis was 5.32 ± 0.77 in heart failure animals that were not treated which is similar to our findings of 5.64 ± 0.49 in hearts from untreated animals with heart failure. Recently, Li et al. have demonstrated that spontaneously hypertensive rats (SHR) with symptoms of heart failure had significantly higher levels of apoptotic myocytes than control myocytes [37]. When treated with an angiotensin-converting enzyme inhibitor, the number of apoptotic cells in the SHR with symptoms of heart failure was dramatically reduced to control levels; controls were SHR without symptoms of heart failure [37]. Kajstura et al. have also reported that angiotensin II increased the percentage of apoptotic cells in isolated adult rat ventricular myocytes, and this effect was mediated by AT-1 receptors [38]. Although ventricular function was not assessed in these studies, the observations raise the possibility that, like our findings with carvedilol, the beneficial effect of ACE-inhibitors or AT-1 receptor blockers in heart failure may in part be attributed to an inhibition of myocyte apoptosis with a resultant improvement in *in vivo* cardiac function, a concept that needs further study.

The data presented suggest that attenuation of apoptosis may underlie the beneficial effect of β-blockade on ventricular function, and that the inhibition of apoptosis can possibly lead to improvement in ventricular function. This represents a novel mechanism to slow the progressive deterioration of myocardial function that can occur in

patients with cardiac failure. Because reactive oxygen radicals play a role in inducing cell apoptosis [39] and the potent antioxidant property of carvedilol [12], we speculate that the beneficial effects attributed to carvedilol (i.e. inhibition of apoptosis and improvement of LV function) are mediated, in part, by its prevention of oxygen-derived free radical damage [35,40]. Our data supports this idea.

Therapeutic Significance

With the clear demonstration that cardiac myocyte apoptosis is present in our heart failure model strategies for prevention of apoptosis can be tested. The clinical consensus has been that higher doses of β-blockade should incur more benefits on ventricular remodeling. However, our studies demonstrated similar benefits at a therapeutic and sub-therapeutic dose. In light of the beneficial effect of carvedilol on the progression of heart failure, and its unique multi-actions that are not shared by any other β-blocking drug or by any other agent currently used in the treatment of heart failure, our findings represent an important new mechanism of improved cardiac function.

The development of animal models of heart failure in which apoptosis is an important feature will allow the modulation of cell death pathways through targeted interventions. Understanding the role of specific signaling pathways in cardiac myocyte apoptosis and developing strategies to manipulate these intracellular pathways may provide in the near future novel therapeutic approaches for the management of heart failure.

Conclusions

In conclusion, our model of heart failure, which shows a high level of congruence with the human condition, has been shown to have a high level of apoptosis that corresponds with cardiac failure. Carvedilol, a non-selective β-blocker with α-blocking properties as well as antioxidant properties, significantly reduced the incidence of programmed cell death while improving cardiac function. This effect was independent of heart rate lowering effects, i.e. β-blockade. The beneficial effects of carvedilol can therefore be obtained with subtherapeutic doses. This is a novel observation and important in treating patients who may not tolerate higher doses (heart rate and blood pressure lowering) of carvedilol. The apoptotic process is linked to the development and progression of heart failure as well as the improvement in cardiac function seen with β-blockade.

Methods

Experimental Design

One day-old broad-breasted white turkey poults, obtained from a commercial breeder, were wing-banded for easy identification. At seven days of age, they were weighed and randomly divided into two groups using a random number generator. The control group (n = 34) was maintained on a normal ration, free from any additives, and the experimental group (n = 91) was fed 700 parts per million furazolidone (Fz) for three weeks. Furazolidone was stopped after day twenty-eight and the animals were randomized into three groups: one group did not receive carvedilol treatment, DCM_0 (n = 52), whereas the other two groups, DCM_1 (n = 19) and DCM_{20} (n = 20), were treated with carvedilol in the same dosages as respective aged-matched control animals (see following). Similarly, age matched control animals were randomized into three groups: one group received no pharmacological agent (Con_0, n = 13), whereas the other two groups received different dosages of carvedilol, 1 mg/kg (Con_1 n = 11) or 20 mg/kg body weight (Con_{20} n = 10). The lower dose of carvedilol did not lower heart rate or blood pressure. However, the higher dose of carvedilol resulted in a significant reduction in heart rate (15%) and blood pressure (9%) for up to eight hours. We performed dose range studies using nine concentrations of carvedilol (data not shown). At the time of euthanasia, body weights, heart weights (after removal of the atria), and LV volumes were obtained on all experimental animals.

Echocardiographic Measurements

Echocardiographic views were obtained using a 7.5 MHz transducer on unsedated and quietly resting animals. Several cardiac cycles were recorded on a videotape and the two dimensional images subsequently played back for analysis. Diastolic left ventricular internal dimension (LVIDd) and systolic left ventricular internal dimension (LVIDs) were also measured and used to determine the fractional shortening. Fractional shortening (%) of the left ventricle was calculated as (LVIDd - LVIDs/ LVIDd) × 100.

TUNEL

Randomly selected hearts from control and cardiomyopathic turkey poults were rapidly removed, flushed with PBS and infused with a tissue embedding medium (Tissue-Tek, OCT Compound, Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. Serial 7 μm cross-sections from heart blocks were cut and fixed to coated slides. Frozen heart sections were fixed in 10% neutral formalin (4% formaldehyde) for 10 minutes at room temperature and post-fixed in Methanol/Acetone (1:1) for 10 minutes at -20°C. Detection of apoptotic cardiac myocytes was achieved by direct immunofluorescence detection of digoxigenin-labeled genomic DNA using the ApopTag Plus in Situ apoptosis detection kit – Fluorescein (Intergen, Purchase, NY). This method used the TUNEL technique to stain DNA fragments in nuclei of apoptotic cells. Tissue sections were then counter-stained with Hoechst 33258 stain (1 μg/ml) (Sigma), and viewed with an epifluorescence microscope (Zeiss Axiophot) equipped with filter sets for fluorescein and Hoechst staining. To quantify

apoptosis, four to five randomly selected microscopic fields per section were examined. The percentage of apoptotic cells was determined by counting the total number of nuclei and TUNEL positive nuclei (apoptotic myocytes). Samples were numbered to conceal the identity of different groups during counting. Sections of interest were photographed using a microscope-integrated 35-mm camera.

Statistical Analysis

Data given in the text are means ± SD. The difference between the means was evaluated using student's *t* test. P < 0.05 was considered significant.

Authors' contributions

CCO drafted and edited the manuscript. AAD carried out creation of the animal model. ECHOs and the echocardiographic measurements were analyzed by NL and JKG. DL, KS and GJ carried out the TUNEL assays. CCO, CPM, RJH and JKG participated in the design of the study. CCO and JKG performed the statistical analysis, and data interpretation. MXL performed figure generation and animal dosing. RJH and JKG conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

Acknowledgements

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