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ORIGINAL ARTICLE

Survival benefit of ghrelin in the heart failure due to dilated cardiomyopathy

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Abstract

Although ghrelin has been demonstrated to improve cardiac function in heart failure, its therapeutic efficacy on the life expectancy remains unknown. We aim to examine whether ghrelin can improve the life survival in heart failure using a mouse model of inherited dilated cardiomyopathy (DCM) caused by a deletion mutation ΔK210 in cardiac troponin T (cTnT). From 30 days of age, ghrelin (150 μg/kg) was administered subcutaneously to DCM mice once daily, control mice received saline only. The survival rates were compared between the two groups for 30 days. After 30-day treatment, functional and morphological measurements were conducted. Ghrelin-treated DCM mice had significantly prolonged life spans compared with saline-treated control DCM mice. Echocardiography showed that ghrelin reduced left ventricular (LV) end-diastolic dimensions and increased LV ejection fraction. Moreover, histoanatomical data revealed that ghrelin decreased the heart-to-body weight ratio, prevented cardiac remodeling and fibrosis, and markedly decreased the expression of brain natriuretic peptide. Telemetry recording and heart rate variability analysis showed that ghrelin suppressed the excessive cardiac sympathetic nerve activity (CSNA) and recovered the cardiac parasympathetic nerve activity. These results suggest that ghrelin has therapeutic benefits for survival as well as for the cardiac function and remodeling in heart failure probably through suppression of CSNA and recovery of cardiac parasympathetic nerve activity.

Introduction

Ghrelin is a growth-hormone – releasing peptide, originally isolated from stomach wall in 1999 (Kojima et al. 1999). In addition to its anabolic effects through the release of growth hormone, ghrelin has been shown to improve cardiac function in heart failure, as indicated by increases in left ventricular (LV) ejection fraction (EF), fractional shortening, and exercise capacity (Nagaya et al. 2001, 2004; Soeki et al. 2008). Recently, several studies have revealed that ghrelin can prevent incidence of malignant arrhythmia in the acute phase of myocardial infarction (MI) (Matsumura et al. 2002; Lin et al. 2004; Soeki et al. 2008; Mao et al. 2012). However, the effect

of ghrelin on survival, which is one of the important indices for heart failure therapy, has not been studied in human beings and even in animal models.

Dilated cardiomyopathy (DCM), a cardiac muscle disorder characterized by cardiac enlargement and systolic dysfunction, is a leading cause of heart failure. Recently, DCM has been found to be caused by a great number of mutations in genes for sarcolemmal transmembrane proteins, cytoskeletal proteins, nuclear envelope proteins, and sarcomeric proteins (Fatkin and Graham 2002; Morimoto 2008). We have created a knock-in mouse model carrying a deletion mutation ΔK210 in the cardiac troponin T (cTnT) gene (Du et al. 2007). The knock-in mice having this mutation develop enlarged hearts with LV systolic dysfunction and premature sudden cardiac death (SCD), well recapitulating a severe early-onset clinical phenotype associated with very frequent SCD and/or heart failure death in human patients (Kamisago et al. 2000; Hanson et al. 2002), and thus being a useful model for evaluating the effect of any drugs on the survival in heart failure (Du et al. 2007; Zhan et al. 2009).

In the present study, we used these knock-in mice as a model of heart failure associated with frequent sudden death and examined whether ghrelin exhibits any beneficial effects on the survival in heart failure. We found that ghrelin had significant survival benefits in heart failure, likely through the suppression of cardiac sympathetic nerve activity (CSNA) and recovery of cardiac parasympathetic nerve activity.

Materials and Methods

Animal model

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animal care was provided in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, which is approved by the Physiological Society of Japan. All protocols were approved by the Animal Subject Committee of the National Cerebral and Cardiovascular Center. In all anesthetised animal experimental protocols, the depth of anesthesia was checked by ensuring that noxious pinch stimulation of the hindpaw, the forepaw, and the ear with blunt forceps did not evoke any motor reflexes.

Knock-in mice with ΔK210 mutation in their endogenous cardiac troponin T gene (*Tnnt2*) on the genetic background of C57BL/6 were used as the DCM model animals (Du et al. 2007). Mixed-gender homozygous mutant and wild-type (WT) mice were obtained by crossing heterozygous mutant mice, and were used as DCM and WT, respectively.

Drug administration

From 30 days of age, ghrelin (150 μg/kg; Peptide Institute, Inc., Osaka, Japan) was administered once daily to DCM mice via a subcutaneous injection (s.c.) for 30 days; control mice received saline only. The dose of ghrelin used in this study was based on the previous studies (Schwenke et al. 2008; Mao et al. 2012) and the source of ghrelin used in this study was rat.

Echocardiography

Echocardiographic studies were performed using an echocardiography system equipped with an 18-MHz phased-array transducer (MS400, VisualSonics Inc., Ontario, Canada) under isoflurane anesthesia.

Histochemistry

After mice were heavily anesthetised intraperitoneally with pentobarbital (50 mg/kg) and butorphanol tartrate (0.4 mg/kg), the hearts were excised and perfused with oxygenated Krebs–Henseleit solution containing 50 mmol/L 2, 3-butanedione monoxime in a Langendorff mode at 37°C as described previously (Du et al. 2007). The hearts were then fixed in 10% formalin neutral buffered solution, cut transversely at the midventricular level, embedded in paraffin, sectioned at 5 mm, and stained with azan. The extent of fibrosis in LV myocardium was quantified using the Image J program from NIH (Bethesda, MD) for three cardiac sections from each mouse.

Heart rate variability analysis

Heart rate (HR) was measured in conscious, unrestrained mice via radio telemetric recording of the electrocardiogram (ECG) (Thireau et al. 2008; Kinoshita et al. 2009). In brief, mice were anesthetised with 2% isoflurane, and a 2-cm vertical incision was made in the skin and abdominal muscle layer by using standard aseptic procedures. The telemetry probe (ETA-F10, Data Sciences International, St. Paul, MN) was placed in the peritoneal cavity. The ECG leads were tunneled under the skin with the tips guided to the right and left axillary regions. The peritoneum and the abdominal skin were then closed with sutures.

The radio telemetric device was implanted at 7 weeks of age. After 1 week of recovery, long-term online recordings of the ECG were digitized and stored for further analysis. Spectral analysis using a fast fourier transformation algorithm on sequences of 1024 points was performed on the HR recordings using the HEM 3.4 software (Notocord Systems, Croissy Sur Seine, France). We selected and analyzed an ECG period lasting for

5 min with no erratic fluctuations, every 120 min during a phase of inactivity (according to the spontaneous telemetry recording of motor activity). Any data that contained ectopic beats or arrhythmic events were deleted manually. Thus, recordings from 12 consecutive time intervals were averaged over a 24-h period. The area under the curve was calculated for the low-frequency (LF: 0.4–1.5 Hz), and high-frequency (HF: 1.5–5.0 Hz) bands, as previously defined for the mouse species (Kinoshita et al. 2009).

Western blot analysis

After briefly perfusing the Langendorff-mounted heart to remove blood from the myocardium, ventricles were dissected from the heart, blotted on filter paper, and homogenized in Laemmli's sample buffer. LV homogenate samples were subjected to western blot analysis as described previously (Nakaura et al. 1999). Expression levels of brain natriuretic peptide (BNP) were determined using an anti-proBNP polyclonal antibody (ab32842; Abcam, Cambridge, UK) and an anti-GAPDH monoclonal antibody (ab9484; Abcam). Signals were visualized using ATTO Chemiluminescence Imaging System (ATTO corporation, Taito-ku, Tokyo, Japan) (Ez-CaptureII) and were quantified using GAPDH signal as a protein loading control.

Statistical analysis

Data are presented as mean ± SEM. Mean values for more than three groups were compared by analysis of variance, followed by a post hoc Tukey's multiple comparison test. Comparisons between two groups were performed with unpaired Student's *t*-test. Survival data utilized the standard Kaplan–Meier analysis. $P < 0.05$ was considered to be significant.

Results

DCM mice with a deletion mutation ΔK210 in the cTnT gene showed a very high mortality due to SCD (Du et al. 2007; Li et al. 2012). Ghrelin significantly improved the life expectancy of DCM mice, with mortality rates being 85% and 55% for treated and untreated DCM mice, respectively, by the 30th day of administration ($P < 0.01$) (Fig. 1).

Heart rate variability (HRV) was determined to assess the balance between sympathetic and parasympathetic tone in conscious DCM mice after 30 days of ghrelin or saline treatment (Fig. 2). The ratio of LF to HF power (LF/HF) and nHF (normalized high-frequency power) was indicative of CSNA and parasympathetic nerve activity, respectively (Thireau et al. 2008; Thayer et al. 2010). DCM mice had significantly higher LF/HF ratio and lower nHF compared to WT mice, indicating an elevated CSNA and a depressed parasympathetic nerve activity. The LF/HF ratio and nHF in ghrelin-treated DCM mice were comparable to those in WT mice, indicating that ghrelin treatment prevented the adverse increase in CSNA and detrimental decrease in cardiac parasympathetic nerve activity in DCM mice.

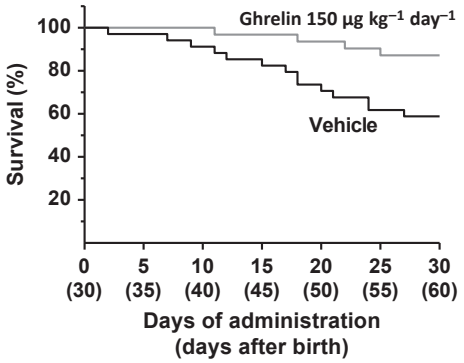

Figure 1. Effect of ghrelin on the survival of a mouse model of inherited dilated cardiomyopathy (DCM) caused by a deletion mutation ΔK210 in the *cTnT* gene. Kaplan–Meier survival curves indicate that mice treated with ghrelin ($n = 31$) have significantly longer life spans compared to mice treated with vehicle only ($n = 34$) (log-rank test, $P < 0.01$)

DCM mice with ΔK210 mutation in cTnT developed markedly enlarged hearts, evident in that the heart-to-body weight ratio was approximately two times higher than that of WT mice (Table 1). This cardiac hypertrophy in DCM mice was associated with significant interstitial fibrosis in the myocardium (Fig. 3) (Du et al. 2007). Ghrelin treatment attenuated the magnitude of cardiac hypertrophy in DCM mice, with the heart weight-to-body weight ratio in ghrelin-treated DCM mice being significantly less than that of untreated DCM mice (Table 1). Ghrelin-treated DCM mice also had significantly less fibrosis formation in the myocardium (Fig. 3).

Echocardiography revealed that, compared to WT mice, DCM mice had markedly increased LV end-diastolic dimension (LVEDD; 4.23 ± 0.21 mm) and reduced LVEF (28.7 ± 3.8%) at 4 weeks of age (Table 2), reflecting significant LV dilation and systolic dysfunction, respectively. Along with the increase in age, LV dilation and systolic dysfunction are progressive (Du et al. 2007). At the age of 8 weeks, LVEDD of DCM mice was 5.42 ± 0.12 mm and LVEF was 25.0 ± 1.6%. Ghrelin treatment decreased the magnitude of ventricular dilation and improved the systolic dysfunction, as indicated by significant decrease and increase in LVEDD and LVEF, respectively.

Consistent with these results, the myocardial expression level of BNP, a biomarker of heart failure, was significantly lower in the ghrelin-treated DCM mice than in the untreated DCM mice (Fig. 4).

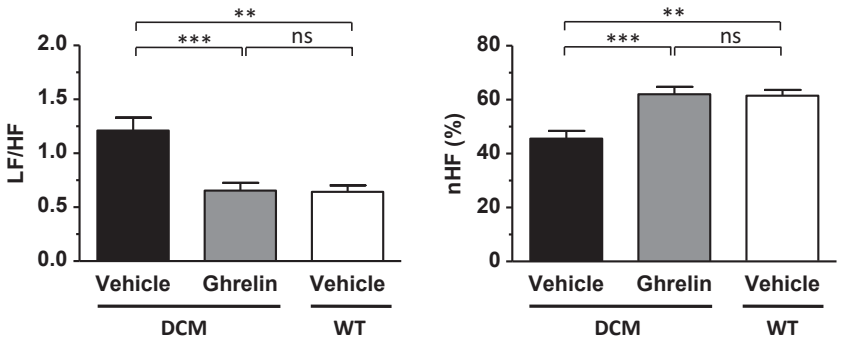

Figure 2. Effect of ghrelin on sympathetic and parasympathetic activities in DCM mice with a deletion mutation ΔK210 in the cTnT gene. HRV was determined to assess cardiac sympathetic nerve activity represented by LF/HF (A) and parasympathetic nerve activity, represented by nHF (B) in WT mice treated s.c. with vehicle(n = 7) and DCM mice treated s.c. with vehicle only (n = 6) or ghrelin (n = 7) once daily for 30 days from 30 days of age. $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1. Heart weight in DCM mice treated with ghrelin.

	WT	DCM	
	Vehicle	Vehicle	Ghrelin
Mice (n)	13	14	16
Age (weeks)	8	8	8
BW (g)	22.93 ± 0.88	22.41 ± 0.89	22.15 ± 0.67
HW (mg)	121.42 ± 3.73	234.73 ± 20.44***	179.93 ± 8.93**,†
HW/BW (mg/g)	5.51 ± 0.27	10.04 ± 0.88***	8.13 ± 0.32**,†

BW, body weight; DCM, DCM mice; HW, heart weight; HW/BW, heart-weight-to-body weight ratio; WT, wild-type.
$^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle-treated WT mice. $^{\dagger}P < 0.05$ versus vehicle-treated DCM mice.

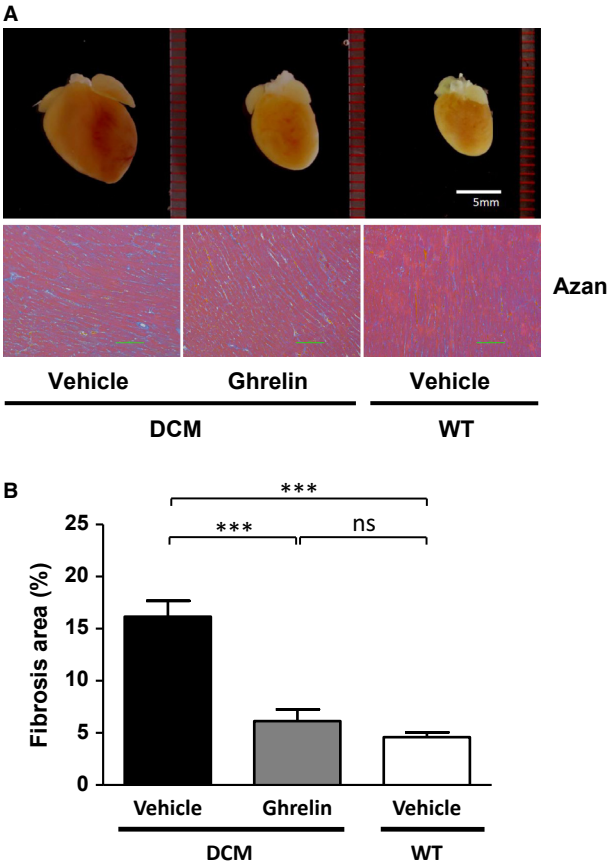

Figure 3. Effect of ghrelin on cardiac remodeling in DCM mice with a deletion mutation ΔK210 in the cTnT gene. Mice were treated s.c. with ghrelin or vehicle only once daily for 30 days from 30 days of age. (A) Gross morphology of the heart (top images; scale bar 2 mm) and histology of the LV myocardium (bottom images; connective tissues were stained blue with azan). (B) Quantitative analysis of the fibrosis area in the LV myocardium (n = 3). $^{***}P < 0.001$.

Discussion

The primary finding of this study is that chronic ghrelin treatment prolongs the life span and improves cardiac remodeling and pump function in mouse model of heart failure due to inherited DCM probably through suppression of excessive CSNA and recovery of cardiac parasympathetic nerve activity.

DCM represents the most prevalent worldwide and leads to significant morbidity and mortality and remains the first cause for cardiac transplantation in the USA (Komajda and Charron 2002; Mohan et al. 2002). Congestive heart failure in DCM patients remains a therapeutic challenge with a 3–5 years mean survival time despite optimal dosages of standard drugs (Perrot et al. 2001), and the ongoing development of novel strategies to prolong the survival are essential for therapy in heart failure. In the present study, we show for the first time that synthetic ghrelin plays a crucial role in reducing mortality, as

Table 2. Echocardiography data in DCM mice treated with ghrelin.

	Baseline		DCM treated with	
	WT	DCM	Vehicle	Ghrelin
Age (weeks)	4	4	8	8
Mice (n)	5	6	8	6
HR (bpm)	422 ± 10	432 ± 11	421 ± 13	414 ± 18
IVSTd (mm)	0.60 ± 0.03	0.52 ± 0.03	0.57 ± 0.03	0.61 ± 0.05
IVSTs (mm)	0.87 ± 0.02	0.65 ± 0.04***	0.73 ± 0.03	0.90 ± 0.06†
LVEDd (mm)	3.16 ± 0.07	4.23 ± 0.21**	5.42 ± 0.12	4.88 ± 0.11††
LVEDs (mm)	2.01 ± 0.22	3.70 ± 0.25***	4.81 ± 0.15	3.98 ± 0.13††
LVPWd (mm)	0.68 ± 0.01	0.69 ± 0.03	0.56 ± 0.04	0.70 ± 0.04†
LVPWs (mm)	1.06 ± 0.05	0.82 ± 0.03**	0.73 ± 0.04	1.01 ± 0.09††
FS (%)	37.0 ± 3.3	13.4 ± 1.9***	11.7 ± 0.8	19.4 ± 1.1†††
EF (%)	67.7 ± 4.6	28.7 ± 3.8***	25.0 ± 1.6	42.5 ± 2.1†††

HR, heart rate; IVST, interventricular septal wall thickness; LV, left ventricular; LVPWT, LV posterior wall thickness; LVEDd, LV end-diastolic dimension; LVEDs, LV end-systolic dimension; LVPWd, LV end-diastolic posterior wall thickness; LVPWs, LV end-systolic posterior wall thickness; FS, fractional shortening; EF, ejection fraction.
**$P < 0.01$, ***$P < 0.001$ versus WT mice. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ versus vehicle-treated DCM mice.

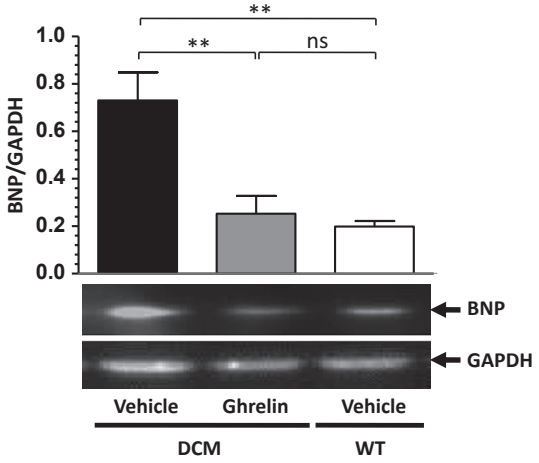

Figure 4. Effect of ghrelin on the expression level of BNP in the LV myocardium of DCM mice with a deletion mutation ΔK210 in the cTnT gene. Mice were treated s.c. with ghrelin or vehicle only once daily for 30 days from 30 days of age and the hearts were excised at 24 h after the last injection to determine the expression levels of pro-BNP ($n = 4$). **$P < 0.01$; ns, not significant.

well as in improving ventricular remodeling and protecting myocardial function in a mouse model of heart failure due to inherited DCM with ΔK210 mutation in cTnT.

Of note is that the mouse model of inherited DCM with ΔK210 mutation in cTnT suffers sudden and frequent ventricular fibrillation (VF) which often proves fatal (Du et al. 2007; Li et al. 2012). In the present study, CSNA, represented by LF/HF, was increased in DCM mice, suggesting that an enhanced cardiac sympathetic tone plays a role in the pathological mechanism in these mice. Several studies have demonstrated that acute administration of ghrelin inhibits sympathetic nerve activity (Matsumura et al. 2002; Lin et al. 2004). Recent studies using animal models of MI have also shown that a single bolus administration of ghrelin prevents arrhythmia and reduces mortality in the acute phase of MI by suppressing CSNA likely through its actions on the vagal afferent nerves (Mao et al. 2012; Schwenke et al. 2012), while repeated bolus administration improves LV dysfunction and attenuates early cardiac remodeling after acute MI (Schwenke et al. 2008; Soeki et al. 2008). On the other hand, parasympathetic nerve activity, represented by nHF, was decreased in DCM mice. We have demonstrated that reduced vagal nervous outflow to the heart plays an important role in VF and SCD occurrence in these DCM mice and that central nervous system-mediated vagal activation is involved, at least in part, in the prevention of VF and SCD by lipophilic β_1-adrenoceptor blocker (Zhan et al. 2009). Our laboratory also has shown that acute central administration of ghrelin activates cardiac vagal nerve and increases dialysate acetylcholine concentrations in the right atrium in anesthetized rabbits (Shimizu et al. 2011). Accordingly, the present data strongly suggest that ghrelin's ability to prevent adverse changes in the autonomic modulation of cardiac function is pivotal for reducing VF and SCD and, thus, improving long-term outcome in this genetic DCM mouse model. However, further studies are required to demonstrate the importance of this mechanism by exploring whether cutting the cardiac vagal nerve does prevent the beneficial effects of ghrelin in the mice.

Hyper-excitation of the sympathetic nervous system unquestionably contributes to progressive cardiac dysfunction and remodeling, and blockade of the β-adrener-

gic receptors attenuates the magnitude of ventricular remodeling seen in heart failure (Sutton and Sharpe 2000; Udelson et al. 2003). Moreover, vagal nerve stimulation has been shown to markedly improve the long-term survival of chronic heart failure rats through the prevention of pumping failure and cardiac remodeling (Li et al. 2004). In the present study, ghrelin treatment improved the cardiac systolic function and attenuated cardiac remodeling in DCM mice, which is likely attributable to ghrelin's ability to modulate sympathetic and parasympathetic control of cardiac function as described above. In addition to this indirect cardioprotective effect via autonomic modulation, ghrelin may also have direct cardioprotective effects on cardiomyocytes as this peptide has been shown to reduce the doxorubicin-induced mortality of cultured H9c2 or endothelial cells (Baldanzi et al. 2002) and the Ara C-induced mortality of cultured HL-1 cells (Iglesias et al. 2004).

Although the mechanism by which subcutaneous administration of ghrelin suppresses CSNA and activates cardiac parasympathetic nerve activity remains unclear, the presence of ghrelin receptors in the vagal nerve terminals of the heart suggests that ghrelin may modulate CSNA, at least in part, through its actions on the vagal afferent nerves terminals (Soeki et al. 2008; Mao et al. 2012). Ghrelin-induced activation of the cardiac vagal afferents, which project to the nucleus of the solitary tract (NTS), would enhance vagal tone and thereby reciprocally decrease CSNA (Soeki et al. 2008). Ghrelin receptors have also been identified centrally in all three divisions of the dorsal vagal complex (Zigman et al. 2006), the hypothalamus including the arcuate nucleus (Guan et al. 1997), and on neurons of the NTS (Lin et al. 2004). A c-Fos expression study reported that centrally administered ghrelin activated the NTS and dorsal motor nucleus of the vagus (Date et al. 2001). Intracisternal administration of ghrelin activated cardiac vagal nerve activity in anesthetized rabbits (Shimizu et al. 2011). Whether the subcutaneously administered ghrelin crosses the blood brain barrier remains to be clearly established. However, the subfornical organ (SFO), a circumventricular structure that lacks the normal blood–brain barrier, has been suggested to be a site through which ghrelin influences the central nervous system (Pulman et al. 2006).

In the present study, we have just demonstrated the survival benefit of ghrelin in a knock-in mouse model of human genetic DCM with a cardiac TnT mutation. Survival effects of ghrelin on DCM with other genetic or nongenetic etiologies remain unknown. However, the present study strongly suggests that ghrelin may be also beneficial for certain forms of DCM that cause frequent VF or sudden death.

In conclusion, the present study reports the therapeutic benefit of ghrelin for survival in heart failure due to inherited DCM with ΔK210 in cTnT, likely through suppression of CSNA and activation of cardiac parasympathetic nerve activity, implicating ghrelin as a promising novel therapeutic strategy for heart failure patients susceptible to VF-induced sudden death.

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Disclosure

None declared.

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