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Abstract

Background Alterations in intracellular Na^+ and Ca^{2+} have been observed in patients with Duchenne muscular dystrophy (DMD) and in animal models of DMD, and inhibition of Na^+-H^+ exchanger 1 (NHE1) by rimeporide has previously demonstrated cardioprotective effects in animal models of myocardial ischemia and heart failure. Since heart failure is becoming a predominant cause of death in DMD patients, this study aimed to demonstrate a cardioprotective effect of chronic administration of rimeporide in a canine model of DMD.

Methods Golden retriever muscular dystrophy (GRMD) dogs were randomized to orally receive rimeporide (10 mg kg^{-1}, twice a day) or placebo from 2 months to 1 year of age. Left ventricular (LV) function was assessed by conventional and advanced echocardiography.

Results Compared with placebo-treated GRMD, LV function deterioration with age was limited in rimeporide-treated GRMD dogs as indicated by the preservation of LV ejection fraction as well as overall cardiac parameters different from placebo-treated dogs, as revealed by composite cardiac scores and principal component analysis. In addition, principal component analysis clustered rimeporide-treated GRMD dogs close to healthy control dogs.

Conclusions Chronic administration of the NHE1 inhibitor rimeporide exerted a protective effect against LV function decline in GRMD dogs. This study provides proof of concept to explore the cardiac effects of rimeporide in DMD patients.

Key words: Canine model of Duchenne muscular dystrophy; echocardiography; left ventricular function; Na^+-H^+ exchanger isoform 1; rimeporide

1. Introduction

Duchenne muscular dystrophy (DMD), a X-linked inherited degenerative muscle disease, occurs in 1/3500 live male. In the past, respiratory failure was the main causes of death for DMD patients**.** In the past two decades, thanks to progress in care, the life expectancy of DMD patients has been considerably extended and heart failure is becoming the predominant cause of death in these patients [1, 2]. Thus, it is of high importance to develop pharmacological strategies against cardiomyopathy in DMD patients.

The Na^+-H^+ exchanger 1 (NHE1) is a transmembrane protein that transports Na^+ into the cell and extrudes H^+, contributing to the regulation of cellular pH, Na^+ and volume. In addition, it is coupled with the Na^+-Ca^{2+} exchanger and participates in the regulation of intracellular calcium. Previous studies showed that inhibition of NHE1 by rimeporide and others is beneficial during myocardial ischemia and heart failure [3-7]. Recently, a 4-week treatment, open label phase Ib, multiple oral ascending dose study enrolled 20 ambulant boys with DMD (6 to 11 years), with outcomes including safety, pharmacokinetic and pharmacodynamic biomarkers [8]. The study was conducted at 4 European sites (London, Milan, Paris and Barcelona). In young DMD boys, rimeporide was safe and well-tolerated at 4 doses ranging from 75 mg/day up to 900 mg/day administered three times a day). Pharmacokinetic evaluations showed that rimeporide was well absorbed orally and that plasma concentrations increased linearly with no evidence of accumulation upon repeated dosing. Exploratory biomarkers of skeletal and cardiac muscle functions and rimeporide mode of action showed positive effect upon a 4-week treatment, supporting its therapeutic potential in patients with DMD and provide the rationale for further efficacy studies (unpublished data). Accordingly, it becomes interesting to hypothesize that rimeporide could exert a beneficial effect in preventing the cardiac functional deterioration in DMD patients, but preclinical proof of concept is needed before attempting any translational approach in

humans. Several arguments are in favor of investigating the cardiac effect of rimeporide in DMD. An increase in intracellular Na^+ concentration has been shown in the skeletal muscle of *mdx* mice and DMD patients [9, 10] and in cardiomyocytes of *mdx* mice [9]. The intracellular Na^+ overload in muscles is accompanied by muscle edema and plays a role in muscle degeneration [10]. This sodium overload is responsible for increased activity of the Na^+-Ca^{2+} exchanger, which leads to disturbed intracellular calcium homeostasis [9, 11, 12]. Indeed, an increase in intracellular calcium at rest or during contraction has been shown in skeletal muscles of DMD patients [13-15] and in cardiomyocytes of *mdx* mice [9, 12, 16]. Considering the close coupling between the Na^+-H^+ exchanger and the Na^+-Ca^{2+} exchanger, inhibiting NHE1 by rimeporide may modify intracellular calcium homeostasis and myocardial function. In this setting, an NHE1 inhibitor prevented the progression of the disease and reduced the mortality of old hamsters with hypertrophic cardiomyopathy and muscular dystrophy [17].

Accordingly, we designed this study to assess cardiac effects of rimeporide in golden retriever muscular dystrophy (GRMD) dogs. Muscular dystrophy that naturally occurs in GRMD dogs shares the same pathogenesis as DMD (i.e., the absence of dystrophin in skeletal and cardiac muscles due to a mutation of dystrophin gene) and has similar pathological patterns. Compared to the *mdx* mouse model of DMD, GRMD dogs develop significant skeletal muscular and cardiac phenotypes similar to those of DMD patients [18-25], which can be accurately assessed by different techniques currently used in humans [21, 22, 24, 25], making it a reliable model for developing therapeutic strategies targeting DMD and obtaining solid preclinical proof of concept.

2. Methods

GRMD dogs were chronically treated with placebo or rimeporide and their left ventricular function was assessed by echocardiography as mentioned in the supplemental methods (Suppl 1).

3. Results

3.1 General information and conventional echocardiography

Throughout the study, there was no death in healthy control dogs. Among 8 placebo-treated GRMD dogs in whom the echocardiographic data were obtained at least at two time points, one died after 4 months of age and two after 9 months while among 8 rimeporide-treated GRMD dogs, two died after 9 months of age, all from non-cardiac cause. The cause of death mainly included pulmonary infection or deterioration of the general condition. Body weight of the animals in the three groups increased with age and no significant difference was detected between rimeporide-treated and placebo-treated GRMD dogs, but their body weights were significantly smaller than those of age-matched healthy control dogs (Table 1). Throughout the study, no obvious adverse reaction related to treatment was observed. Heart rate of the animals in the three groups decreased with age and there was no difference between rimeporide- and placebo-treated GRMD dogs but from 6 months of age, GRMD dogs had a slightly but significantly higher heart rate than healthy control dogs (Table 1).

To assess the evolution of LV function, we longitudinally performed conventional and advanced echocardiography in the three groups of animals. Table 1 shows the main cardiac parameters measured by conventional echocardiography. LV wall thickness increased with age in the 3 groups of animals as indicated by end-diastolic interventricular septal thickness and end-diastolic posterior wall thickness, but at the same age, GRMD dogs had a thinner LV wall, especially at the interventricular septum, compared to healthy control dogs. However, when these parameters were corrected by body weight, such difference disappeared (Table 1).

There was no significant difference in both interventricular septal and posterior wall systolic thickenings among the three groups. Both LV end-diastolic and end-systolic diameters increased significantly with age in the 3 groups. There was no significant difference among the 3 groups, although these two parameters tended to be greater in placebo-treated GRMD dogs at 1 year of age. Fractional shortening was significantly decreased in placebo-treated GRMD dogs starting from 9 months, whereas in rimeporide-treated GRMD dogs it remained similar to healthy control dogs at all ages (Table 1). To more accurately appreciate changes in LV dimension and overall function, we measured LV volume in both apical 4C and 2C views using the biplane method of disks (modified Simpson's rule) and calculated LV fraction ejection. As shown in Table 1, at 2 months of age, both rimeporide- and placebo-treated GRMD dogs had a smaller LV end-diastolic volume than healthy control dogs (both $p<0.05$), and LV end-diastolic volume increased significantly with age. However, there was a trend that this increase was less important in rimeporide-treated GRMD than in placebo-treated GRMD dogs starting from 9 months of age. A similar trend was also observed for LV end-systolic volume. The changes in LV end-diastolic and end-systolic volumes resulted in a significant decrease in LV ejection fraction in placebo-treated GRMD dogs at 9 months of age, indicating degradation of LV overall function while LV ejection fraction remained overall stable or slightly modified in rimeporide-treated GRMD dogs. Calculated cardiac output and stroke volume were significantly higher in healthy control dogs than in GRMD dogs of the same age, but these parameters changed with age in the same manner in the 3 groups of animals.

The ratio of peak early diastolic filling velocity (E wave) to peak late filling velocity at atrial contraction (A wave) of transmitral inflow is a widely used parameter to assess LV diastolic function. As shown in Table 1, the E/A ratio remained stable in the 3 groups of animals during the study period and there was no significant difference among the 3 groups.

Vp during early filling, another index of LV diastolic function, showed improvement under rimeporide with a significant difference compared with placebo-treated GRMD dogs. Rimeporide-treated GRMD dogs showed an increasing trend of Vp with age, while Vp values in the placebo-treated GRMD dogs showed an increase until 6 months of age and a decrease from 9 months.

3.2 Changes in LV myocardial strain analyzed by speckle-tracking echocardiography

Thanks to the characteristic of independence on the Doppler angle of incidence [26], speckle–tracking echocardiography has emerged as a reliable technique for the assessment of regional and overall myocardial function and this technique has been proposed to detect early LV dysfunction in DMD patients [27, 28] and in GRMD dogs [25]. Therefore, to examine the effect of the treatment with rimeporide on LV myocardial strain, longitudinal strain and strain rate were analyzed in 3 apical views. As shown in Table 2, at the apical 2C view, there was a significant change in longitudinal strain with age, which was particularly apparent for placebo-treated GRMD dogs from the age of 9 months, while rimeporide-treated GRMD dogs had a stable longitudinal strain until 9 months. Healthy control dogs had a stable longitudinal strain throughout the study period. A similar trend was also observed in longitudinal strain obtained at the apical 3C and 4C views. Longitudinal strain rate obtained in the 3 apical views decreased with age in 3 groups of animals, but such decrease was more visible in placebo-treated GRMD dogs from the age of 6 months at the apical 2C view (Table 2). In contrast to healthy control dogs in whom no dyskinetic segment was observed, a larger number of dyskinetic segments (defined by a longitudinal strain value > -10%) were observed in placebo-treated GRMD dogs at 9 months of age. This was apparent at the apical 2C view at which 4/7 of placebo-treated GRMD dogs had at least one dyskinetic segment with a total of 10 dyskinetic segments. Interestingly, no dyskinetic segments were observed in rimeporide-treated GRMD dogs at 9 months of age. Throughout the study (from 2 months to 12 months),

placebo-treated GRMD dogs had an average number of 1.7 dyskinetic segments/dog, while rimeporide-treated GRMD dogs had only 0.3 dyskinetic segments/dog (p = 0.054, Wilcoxon rank sum test with continuity correction, two-sided).

LV twist-untwist mechanics are an important aspect of LV function and can be analyzed by speckle-tracking echocardiography [29]. Therefore, we analyzed LV twist and untwisting mechanics in the 3 groups of animals (Table 2). In placebo-treated GRMD dogs, LV twist decreased with age, whereas it remained stable in rimeporide-treated GRMD dogs as in healthy control dogs, suggesting a preserved LV twist mechanic (i.e., systolic function) in rimeporide-treated GRMD dogs. LV twisting rate decreased with age in the 3 groups of dogs. However, it appeared that such a reduction was slower in rimeporide-treated GRMD dogs, suggesting an improved LV twist mechanic in these dogs. Finally, regarding LV untwisting rate, an important determinant of early diastolic function of the left ventricle [30], it was increased or maintained stably in healthy control dogs and in rimeporide-treated GRMD dogs with increasing age but it was slightly but not significantly decreased in placebo-treated GRMD dogs at 1 year of age, suggesting that LV diastolic function remained in the normal range in both rimeporide-treated and placebo-treated GRMD dogs at this age.

3.3 Composite cardiac scores and PCA

To further evaluate the therapeutic effect of rimeporide, a set of LV dimensional and functional parameters reflecting LV regional and global functions including LV end-diastolic and end-systolic diameters and volumes, fractional shortening, ejection fraction, longitudinal strain obtained at 2C and 4C views and Vp was selected for the calculation of composite cardiac scores and PCA. A special attention was paid to perform such analyses in the conditions where there was a maximal effective number and LV functional parameter started to change in GRMD dogs. The 9 months of age met such conditions. The results from the analysis of composite cardiac scores and PCA indicated that rimeporide-treated GRMD dogs

exhibited overall cardiac parameters different from those of placebo-treated GRMD dogs: the majority of rimeporide-treated GRMD dogs were clustered close to healthy control dogs whereas no placebo-treated GRMD dogs were clustered close to healthy control dogs (Fig. 1 and Fig. 2). These results suggested that rimeporide can limit the decline of LV function in GRMD dogs, whereas placebo did not produce such effect.

4. Discussion

Because dystrophin-deficient dilated cardiomyopathy occurs in DMD patients and is becoming the leading cause of death in DMD patients, it is recommended to treat cardiac involvement as early as possible [1, 2, 31]. This preclinical prospective study examined the effect of rimeporide on the evolution of LV dimensional and functional parameters measured by echocardiography. Compared to placebo-treated GRMD dogs showing a decline in LV ejection fraction at the age of 9 months, rimeporide-treated GRMD dogs had a preserved LV ejection fraction. Thus, rimeporide exerted a cardioprotective effect by preventing LV dilation. This can be explained by its effect on NHE1 to prevent the intracellular Na^+ accumulation seen in DMD patients and *mdx* mice [9, 10]. In addition, contrasting with placebo-treated GRMD dogs that had a decreased longitudinal strain (particularly at the apical 2C view) from 9 months of age and showed a large number of dyskinetic segments, rimeporide-treated GRMD dogs showed a maintained longitudinal strain and a smaller number of dyskinetic segments throughout the study period. This protection against LV dysfunction by rimeporide is in accordance with a study in hamsters with hereditary cardiomyopathy showing a significant preventive effect of NHE1 inhibition on cardiac necrosis [17]. Finally, rimeporide-treated GRMD dogs showed a preserved LV twist like in healthy control dogs as opposed to placebo-treated GRMD dogs which showed a decreased LV twist with age. The maintained LV twist mechanics may also contribute to preserving LV overall function in rimeporide-treated GRMD dogs. Interestingly, analysis using composite cardiac scores and PCA showed that the majority of 9-month-old rimeporide-treated GRMD dogs had positive composite cardiac scores and clearly clustered them close to healthy control dogs (Fig. 1 and Fig. 2). The composite cardiac score is a relative score (Z-score), centered on 0 by default. A negative value does not mean that the dog did not respond, it indicated that the dog responded less than the average of the dogs in the study. In Fig. 1, 3

groups can be observed: 4 placebo-treated dogs on the left who had the worst responses, rimeporide-treated dogs in the center who had average response (with 2 placebo-treated dogs in the middle, who had probably less severe phenotype), and healthy control dogs on the right. Altogether, these results clearly indicated a difference in the trajectory of decline in LV dysfunction and a cardioprotective effect of rimeporide in GRMD dogs. It may be worth noting that in parallel to the evaluation of cardiac effects of rimoporide, the effects of rimeporide on skeletal muscle function have been examined in these GRMD dogs, and the results are to be reported elsewhere.

Although rimeporide appears to exert cardioprotective effect through regulating intracellular Na^+, H^+ and Ca^{2+}, it does not restore dystrophin or cardiomyocyte attachment and integrity. Thus, it remains unknown whether damage to cardiomyocytes is still a problem, even with rimeporide treatment. However, it was demonstrated that the transgenic over-expression of the canine Na^+-Ca^{2+} exchanger in skeletal muscles of both δ sarcoglycan-null and dystrophin-null (*mdx*) mice exacerbated the dystrophic phenotype [32], and in *mdx* mice and cardiomyopathic hamsters that have an increased NHE-1 activity, NHE-1 inhibition significantly reduced both Na^+ and Ca^{2+} overload and improved muscle function [33]. In addition, in our previous study performed in GRMD dogs [19], chronic bradykinin treatment improved cardiomyocyte function. If this is also the case for rimeporide, a cardiomyocyte protection by rimeporide would be expected. Clearly, further cellular investigations are needed to clarify this issue.

This study may have limitations. Despite this beneficial effect observed in rimeporide-treated GRMD dogs at 9 months of age, the difference between rimeporide-treated and placebo-treated GRMD dogs became less evident and not significant at 1 year of age due to an insufficient number of animals after death from other reasons than cardiac issues. In this study, the animals were only followed up until 1 year when mild LV dysfunction could be

observed, thus, it is difficult to predict the effect of rimeporide in the late stage where LV function is more severely altered. However, based on previous reports [3, 6, 7, 17], it is reasonable to speculate that this beneficial effect will last and even be amplified in GRMD dogs presenting more severe LV dysfunction. The pharmacokinetics of rimeporide has been studied in healthy volunteers, DMD patients [8] and dogs (beagle and GRMD). The short half-life justifies 2-3 administrations day. In toxicology studies, beagle dogs received rimeporide up to 75 mg/kg for 39 weeks with no signs of acute or chronic toxicity (unpublished data). In patients, the treatment was given 3 times a day up to 900 mg/day (3 times 300 mg) without adverse events. However, for feasibility reasons, the GRMD dogs received 2 doses of 10 mg/kg at around 8 am and 6 pm. As there is no restriction of safety with rimeporide, it is reasonable to speculate that a higher dose of rimeporide would probably lead to better outcomes. We did not calculate the relative change in parameters from time point to time point, considering that it may be not physiologically relevant because the age period from 2 months to 9-12 months represents an important development phase for a dog, *i.e.*, from puppy to adult, in which important physiological modifications have occurred.

In conclusion, chronic inhibition of NHE1 with oral rimeporide afforded protection against functional deterioration of the left ventricle in GRMD dogs. Because of the similarity in the pathogenesis and pathological changes between GRMD and DMD, our study provides proof of concept to support a translational approach by exploring the cardiac effects of rimeporide in DMD patients.

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Conflict of interest

F. Porte Thomé and T. D. Tran are employees of the EspeRare, a not-for-profit foundation. F. Porte Thomé is a co-founder of the EspeRare foundation. Employees and founders of the EspeRare Foundation do not own stock or options. The patent on rimeporide has expired and these results will not lead to patent prosecution. The other authors have nothing to declare.

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Figure captions

Fig. 1 Representative images from echocardiography of control and GRMD dogs and distribution of composite cardiac scores at 9 months of age

A. M-mode echocardiographic images from a parasternal short-axis view showing reduced fractional shortening (FS) in a placebo-treated GRMD dog (middle panel) and normal FS in a rimeporide-treated GRMD (right panel) compared to a healthy control (left panel). LVPW: left ventricular posterior wall; IVS: interventricular septum. B. Distribution of composite cardiac scores. In contrast to the majority of placebo-treated GRMD dogs (5/7) who had negative composite cardiac scores and placed far away those of healthy control dogs, four out of eight rimeporide-treated GRMD dogs had positive composite cardiac scores and clustered close to those of healthy. The abscissa axis shows the names of dogs.

Fig. 2 Calculation of LV longitudinal strain from the apical 4-chamber view and principal component analysis of cardiac parameters of the 3 groups of dogs at 9 months of age

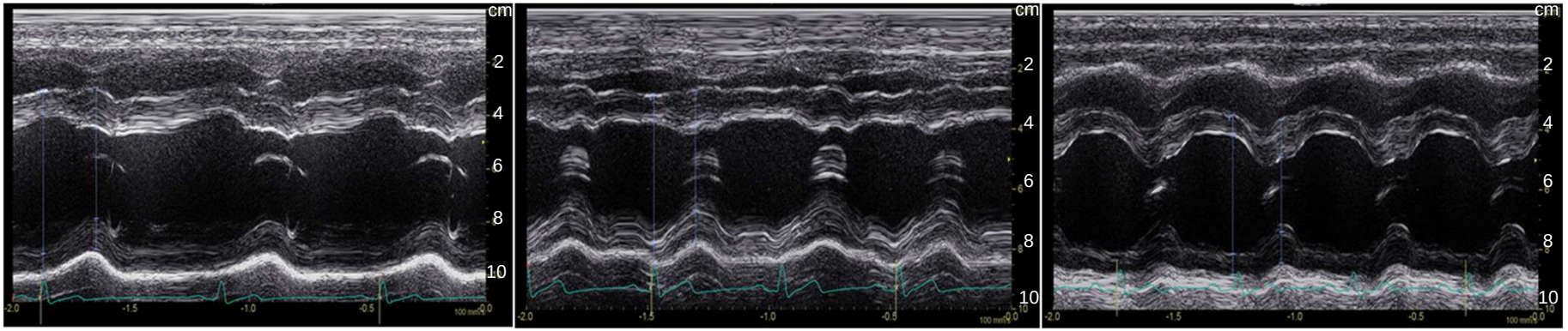
A. Speckle tracking analysis of longitudinal strain from the apical 4-chamber view showing an altered global strain (GS) in a placebo-treated GRMD dog (middle panels) and normal GS in a rimeporide-treated GRMD (right panels) compared to a healthy control (left panels). B. Principal component analysis of cardiac parameters. The two first principal components explained 75% of the variance. Dogs are colored as follows: rimeporide-treated GRMD in blue, placebo-treated GRMD in black and healthy control dogs in green. Red dashed arrows indicate the projection of the cardiac parameters on the two first principal component axes. The names of dogs are showed in the figure.

A

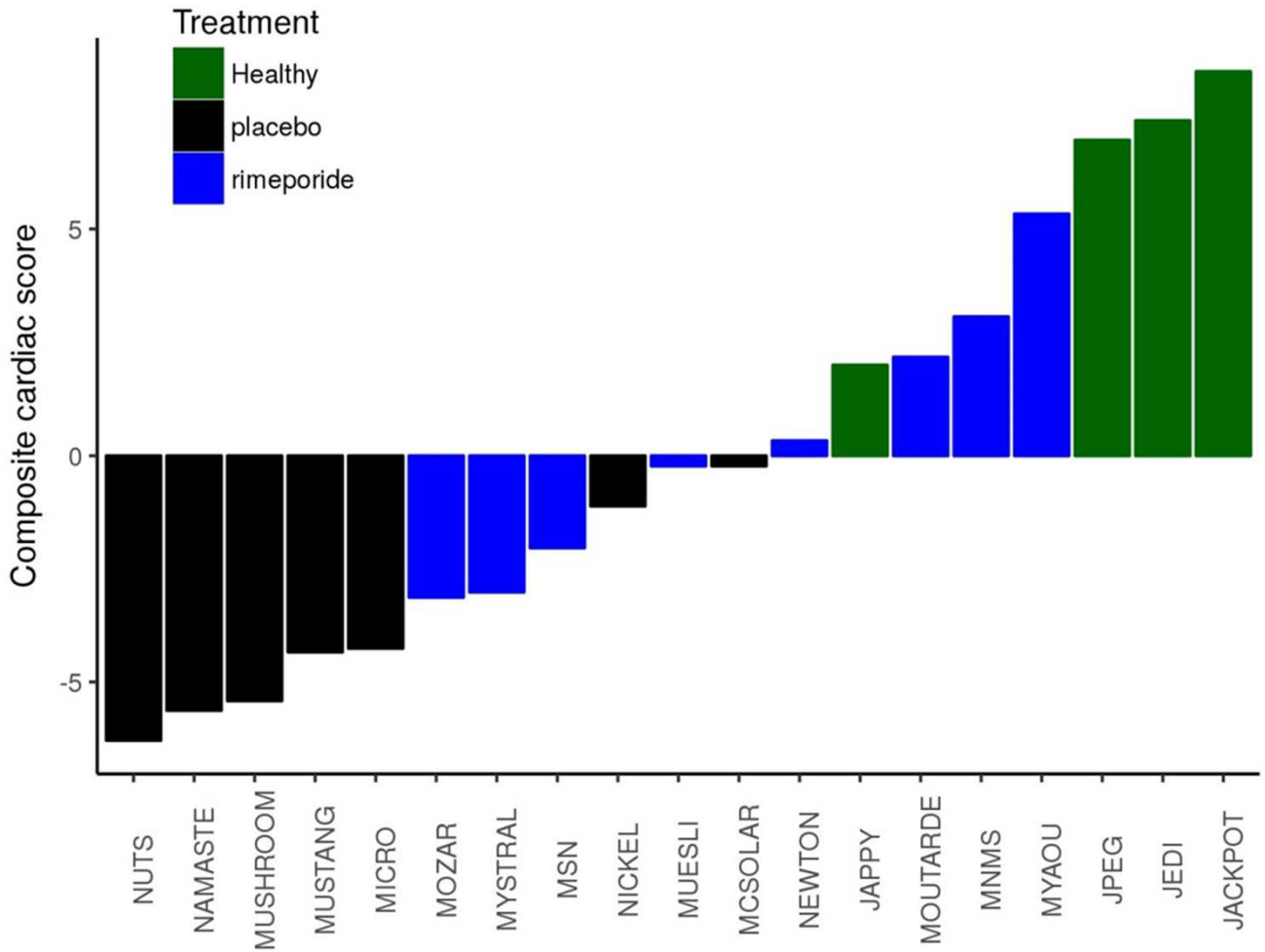
Healthy control dog
FS= 37%

Placebo-treated GRMD dog
FS= 32%

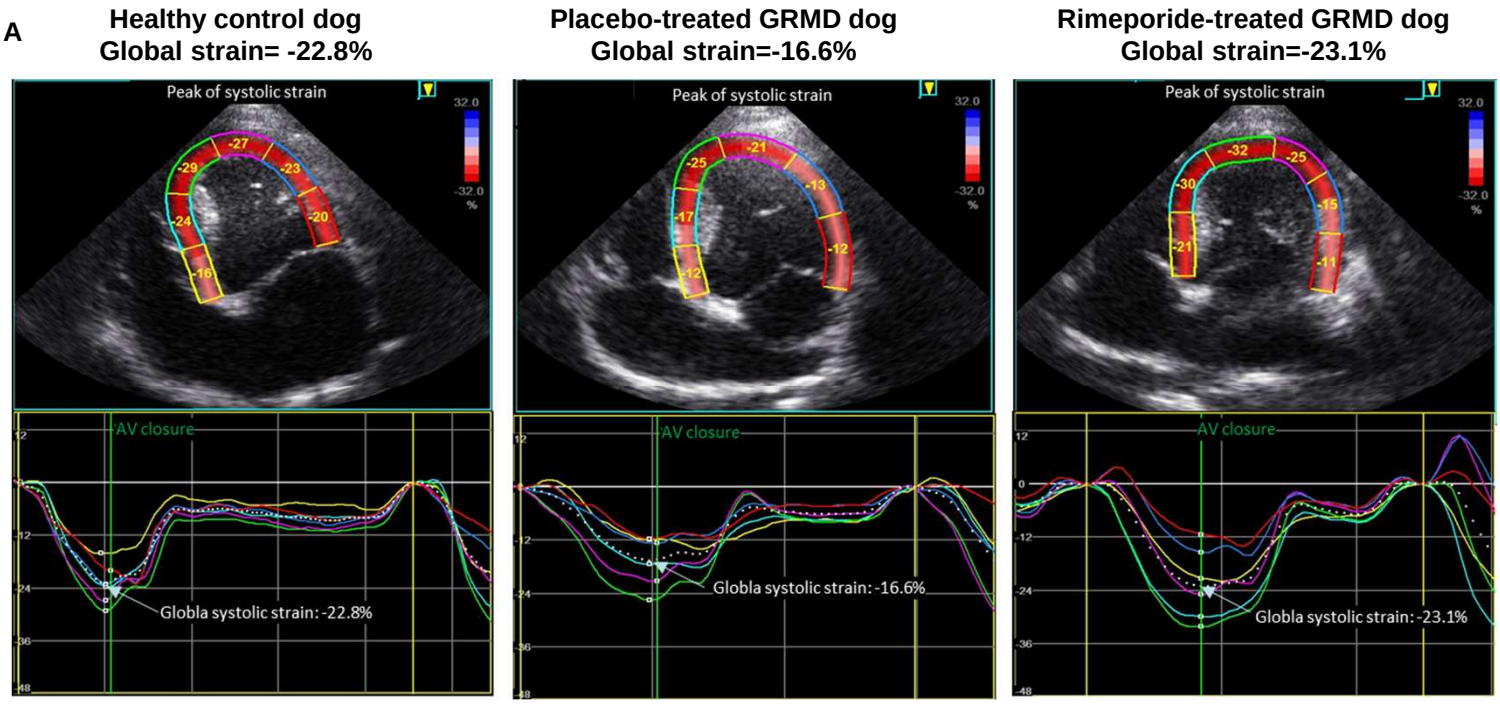
Rimeporide-treated GRMD dog
FS= 37%



B



A


Healthy control dog
Global strain= -22.8%
Placebo-treated GRMD dog
Global strain=-16.6%
Rimeporide-treated GRMD dog
Global strain=-23.1%
Peak of systolic strain
AV closure
Globla systolic strain: -22.8%
Globla systolic strain: -16.6%
Globla systolic strain: -23.1%

B

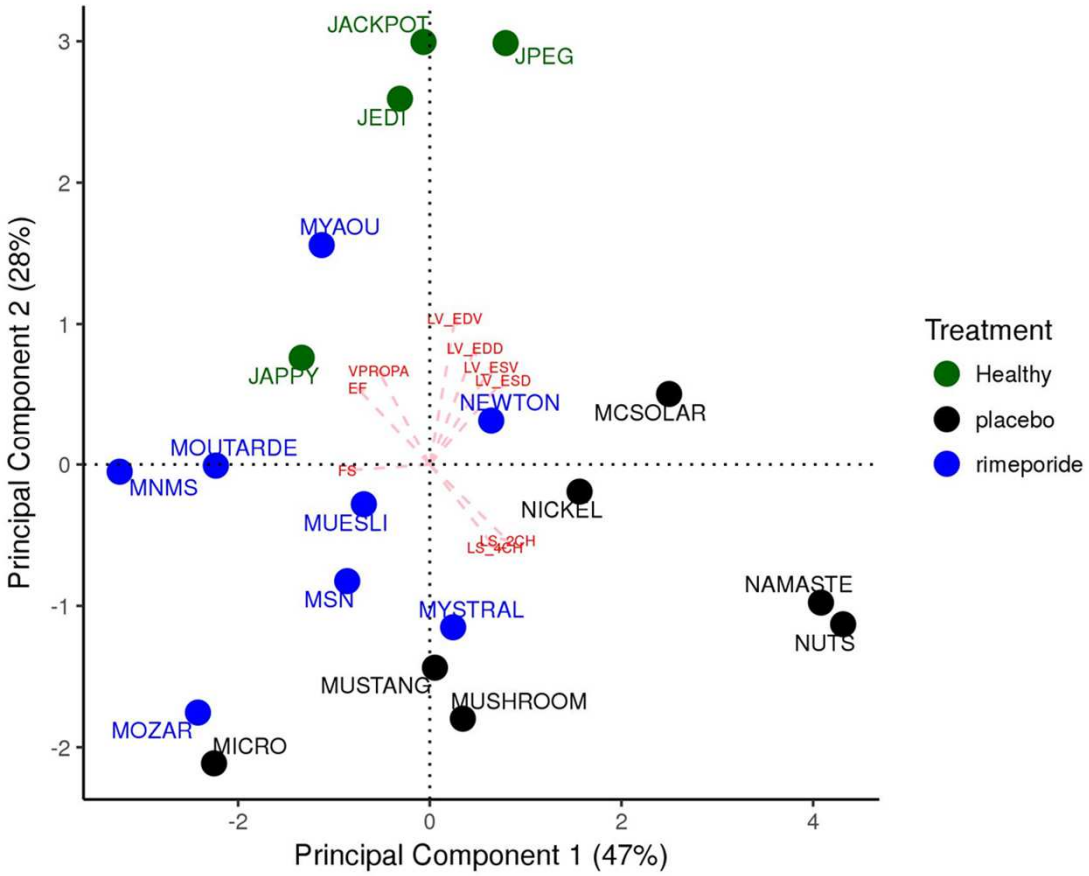

JACKPOT
JPEG
JEDI
MYAOU
JAPPY
VPROPA
EF
LV_EDV
LV_EDD
LV_ESV
LV_ESD
NEWTON
MCSOLAR
MOUTARDE
MNMS
FS
MUESLI
NICKEL
LS_2CH
LS_4CH
MSN
MYSTRAL
NAMASTE
NUTS
MUSTANG
MUSHROOM
MOZAR
MICRO
Principal Component 2 (28%)
Principal Component 1 (47%)
Treatment
Healthy
placebo
rimeporide

Table 1 Comparison of body weight, heart rate and LV functional parameters by conventional echocardiography and Doppler techniques

Parameter	Group	Age (months)					ANOVA *P* value	
		2	4	6	9	12	time	ARM
BW	Rimeporide	3.2±0.3†	10.6±0.9	15.7±1.0†	18.4±1.3†	20.7±1.4†		
(kg)	Placebo	3.0±0.2†	10.2±0.8	15.3±0.9†	19.4±1.6†	21.5±1.3†	4.9e-66	0.9209
	Control	4.5±0.7	12.9±1.6	21.4±1.0	27.5±2.2	29.5±1.6		
Heart rate	Rimeporide	166±6	149±6	132±8†	105±5	111±9†		
(beats min^{-1})	Placebo	168±7	147±7	116±6	104±3	102±5	1.6e-28	0.0229
	Control	165±5	138±7	100±7	94±4	84±2		
EDIVST	Rimeporide	4.6±0.2	6.6±0.2	7.0±0.5	7.0±0.5†	7.0±0.8†		
(mm)	Placebo	4.5±0.3	6.8±0.2	6.8±0.4	7.1±0.4†	6.6±0.3†	2.0e-8	0.8657
	Control	5.4±0.6	7.5±0.6	8.4±0.9	10.0±1.0	11.4±1.0		
EDIVST/BW	Rimeporide	1.5±0.2	0.7±0.1	0.4±0.0	0.4±0.0	0.3±0.0		
(mm kg^{-1})	Placebo	1.5±0.1	0.7±0.0	0.4±0.0	0.4±0.0	0.3±0.0	1.3e-21	0.8692
	Control	1.2±0.1	0.6±0.1	0.4±0.1	0.4±0.1	0.4±0.1		
IVS	Rimeporide	66±7	56±5	68±7	65±7	76±16		
thickening	Placebo	46±5	44±5	61±3	59±7	52±13	0.1047	0.8761
(%)	Control	56±9	65±13	59±11	65±11	53±10		
EDPWT	Rimeporide	4.3±0.3	5.4±0.3	6.7±0.3	7.5±0.3	7.0±0.2†		
(mm)	Placebo	4.0±0.2	6.2±0.3	6.9±0.4	7.6±0.5	7.6±0.5†	3.3e-21	0.3751
	Control	4.2±0.3	6.6±0.6	7.5±0.6	7.6±0.3	9.3±.08		
EDPWT/BW	Rimeporide	1.4±0.1	0.5±0.1	0.4±0.0	0.4±0.0†	0.3±0.0		
(mm kg^{-1})	Placebo	1.4±0.1	0.6±0.0	0.4±0.0	0.4±0.0†	0.4±0.0	3.6e-18	0.7053

	Control	1.0±0.2	0.5±0.1	0.4±0.0	0.3±0.0	0.3±0.0		
PW	Rimeporide	50±7*	74±9	70±7	59±5†	57±12		
thickening	Placebo	74±6	55±7	67±8	49±5†	58±8	0.2218	0.7740
(%)	Control	70±9	66±6	74±13	74±5	59±10		
End-diastolic	Rimeporide	2.2±0.1	3.1±0.1	3.5±0.2†	4.2±0.2	4.6±0.1		
diameter	Placebo	2.0±0.1	2.8±0.2	3.4±0.2†	4.2±0.2	4.9±0.4	3.3e-79	0.7989
(cm)	Control	2.3±0.2	3.3±0.3	4.2±0.2	4.4±0.3	4,6±0.3		
End-systolic	Rimeporide	1.4±0.1	2.0±0.1	2.2±0.1†	2.6±0.1	3.0±0.2		
diameter	Placebo	1.3±0.1	1.8±0.1	2.1±0.1†	2.8±0.2	3.4±0.4	4.8e-59	0.5463
(cm)	Control	1.5±0.2	2.2±0.2	2.6±0.2	2.8±0.2	3.0±0.3		
Fractional	Rimeporide	36.9±1.3	36.2±1.2	39.2±1.4	38.3±1.4	34.4±1.4		
shortening	Placebo	36.8±0.7	36.1±0.8	38.0±2.1	33.8±1.7	30.1±3.0	0.0094	0.0949
(%)	Control	37.4±3.0	35.7±3.2	38.5±1.7	35.3±1.6	35.2±1.6		
End-diastolic	Rimeporide	6.5±0.5†	21.4±1.3†	27.5±3.3†	36.9±3.4†	46.1±6.3†		
volume	Placebo	6.8±.7†	19.1±2.4†	28.5±1.7†	41.8±2.4†	54.0±5.0	7.2e-98	0.5936
(ml)	Control	11.8±1.1	32.5±5.7	49.1±5.0	63.4±4.5	66.6±4.7		
End-systolic	Rimeporide	2.5±0.2†	8.3±0.6†	10.8±1.3†	14.2±1.6†	20.8±3.0		
volume	Placebo	2.6±0.3†	7.8±1.3†	11.5±0.7†	18.7±1.6	25.7±3.3	5.0e-77	0.2518
(ml)	Control	4.7±0.1	12.5±0.3	16.4±0.3	22.1±0.3	27.5±0.7		
Ejection	Rimeporide	61.1±1.0	61.2±1.6	60.4±1.4†	61.7±1.4*	55.0±1.5		
fraction	Placebo	61.9±1.6	60.2±1.6	59.8±1.4†	54.7±2.1†	52.6±3.5	2.1e-6	0.0329
(%)	Control	60.2±2.3	61.8±2.5	66.3±3.1	64.3±2.8	59.0±2.1		
Cardiac	Rimeporide	0.6±0.1†	1.9±0.1†	2.1±0.2†	2.3±0.2†	2.7±0.4		
output	Placebo	0.7±0.1†	1.6±0.2†	2.0±0.1†	2.3±0.2†	2.8±0.4	1.6e-30	0.6321
(l min^{-1})	Control	1.1±0.1	2.7±0.3	3.0±0.4	3.3±0.4	3.5±0.7		

Stroke	Rimeporide	4.0±0.3†	13.1±0.8†	16.7±2.1†	22.7±1.9†	25.3±3.5†		
volume	Placebo	4.2±0.5†	11.4±1.3†	17.1±1.1†	22.2±0.8†	28.2±2.8†	4.9e-73	0.9136
(ml)	Control	7.0±0.6	19.0±1.6	32.4±3.8	35.8±3.0	41.8±5.1		
E/A ratio	Rimeporide	1.4±0.1	1.3±0.1	1.3±0.1	1.2±0.0	1.3±0.1		
	Placebo	1.3±0.1	1.3±0.1	1.3±0.1	1.3±0.1	1.3±0.2	0.3978	0.6750
	Control	1.3±0.1	1.3±0.2	1.3±0.1	1.4±0.1	1.4±0.2		
Vp	Rimeporide	62±3†	65±3	71±3	69±2*	69±6†		
(cm s^{-1})	Placebo	63±2†	70±3	67±3	56±3†	54±4†	0.6183	0.0414
	Control	81±24#	75±6	76±3	78±9	86±7		

Data are presented as the mean ± SEM. For rimeporide group, n=8, 8, 8, 8 and 6 at 2, 4, 6, 9 and 12 months of age; for placebo group, n=8, 8, 7, 7 and 5 at the age of 2, 4, 6, 9 and 12 months; for control group, n= 4 at all ages (except Vp that was measured in only 2 control dogs at 2 months). ANOVA *p*-values consider the overall differences across all time points (see Methods). The significance of the treatment arm (ARM: rimeporide versus placebo) was assessed with a type II ANOVA. In addition, one-way ANOVA was performed for each age, and when an overall difference was detected, a Student-Newman-Keuls test was performed to make pairwise comparison. * $p<0.05$ versus rimeporide-treated group, and † $p<0.05$ versus healthy control group. BW: body weight; E/A ratio: **t**he ratio of peak flow velocity in early diastole (E wave) to peak flow velocity in late diastole (A wave); EDIVSWT: end-diastolic interventricular septal wall thickness; EDPWT: end-diastolic posterior wall thickness; IVS thickening: interventricular septal systolic thickening; PW thickening: posterior wall systolic thickening; Vp: flow propagation velocity

Table 2 Comparison of left ventricular strain, strain rate, twist and untwisting analyzed by speckle-tracking echocardiography

Parameter	Group	Age (months)					ANOVA *P* value	
		2	4	6	9	12	time	ARM
Longitudinal strain (2C view, %)	Rimeporide	-20.7±1.2	-18.6±1.1	-19.6±0.9	-19.8±1.1*	-16.8±1.0†		
	Placebo	-19.4±0.4	-18.9±0.7	-19.5±0.6†	-17.7±1.7†	-16.1±1.0†		
	Control	-20.2±0.6	-19.9±0.4	-20.5±0.6	-21.8±0.9	-22.1±1.4	4.2e-6	0.0077
Longitudinal strain (3C view, %)	Rimeporide	-19.9±1.0	-20.5±1.0	-18.6±0.4	-20.1±0.7	-17.6±0.8†		
	Placebo	-19.4±0.4	-18.9±0.7	-19.5±0.6	-17.7±1.7	-16.1±1.0†		
	Control	-20.2±0.6	-19.9±0.4	-20.5±0.6	-21.8±0.9	-22.1±1.4	0.0022	0.0911
Longitudinal strain (4C view, %)	Rimeporide	-19.3±0.5†	-20.3±0.7*	-20.7±0.7	-20.8±0.7*	-18.6±1.8		
	Placebo	-20.1±0.6†	-19.2±0.5†	-17.6±0.2	-15.2±1.1†	-15.8±0.7		
	Control	-22.2±0.3	-19.6±0.8	-20.8±1.1	-21.2±0.4	-21.8±1.3	0.3187	0.0121
Longitudinal strain rate (2C view, s^{-1})	Rimeporide	-2.7±0.2†	-2.2±0.2	-2.2±0.1*	-2.0±0.2	-1.7±0.2		
	Placebo	-2.4±0.1†	-2.1±0.2	-1.7±0.1†	-1.7±0.2	-1.4±0.2	4.4e-11	0.0307
	Control	-3.2±0.1	-2.3±0.2	-2.1±0.1	-2.2±0.1	-2.0±0.3		
Longitudinal strain rate (3C view, s^{-1})	Rimeporide	-2.8±0.1	-2.5±0.1	-2.0±0.1	-2.2±0.2	-1.9±0.1†		
	Placebo	-2.6±0.2	-2.2±0.1	-2.6±0.2	-1.8±0.3	-1.7±0.2†		
	Control	-2.8±0.4	-2.2±0.2	-1.9±0.3	-2.1±0.2	-2.3±0.2	3.1e-7	0.3861
Longitudinal strain rate (4C view, s^{-1})	Rimeporide	-2.8±0.1†	-2.5±0.1†	-2.4±0.1	-2.3±0.1	-2.0±0.2		
	Placebo	-2.5±0.2†	-2.2±0.1†	-2.6±0.2	-1.9±0.1	-1.8±0.1		
	Control	-3.2±0.5	-3.1±0.2	-2.6±0.3	-2.3±0.1	-2.5±0.4	1.7e-7	0.4142
LV twist (°)	Rimeporide	10.6±1.2	13.4±1.5	11.1±0.7	10.2±1.4	10.3±1.6		
	Placebo	12.9±0.9	10.0±0.7	11.1±1.5	9.7±0.8	7.3±1.4†	0.0003	0.1278

	Control	12.7±0.6	11.6±0.5	12.7±0.8	12.4±0.9	12.1±0.5		
LV twisting	Rimeporide	185±28	158±14	153±16	147±14	154±25		
rate	Placebo	200±20	134±20	128±10	129±14	102±15	1.6e-7	0.2209
(° s^{-1})	Control	188±23	161±29	131±7	121±5	108±20		
LV	Rimeporide	-122±25	-143±18	-144±19	-118±22	-121±20		
untwisting	Placebo	-110±20	-115±20	-131±22	-106±21	-94±23	0.6860	0.1019
rate (° s^{-1})	Control	-125±7	-164±26	-149±17	-116±15	-149±17		

Data are expressed as the mean ± SEM. For rimeporide group, n=8, 8, 8, 8 and 6 at age of 2, 4, 6, 9 and 12 months; for placebo group, n=8, 8, 7, 7 and 5 at age of 2, 4, 6, 9 and 12 months; for control group, n= 4 at all ages. ANOVA *p*-values consider the overall differences across all time points (see Methods). The significance of the treatment arm (ARM: rimeporide versus placebo) was assessed with a type II ANOVA. In addition, one-way ANOVA was performed for each age, and when an overall difference was detected, a Student-Newman-Keuls test was performed to make pairwise comparison. * $p<0.05$ versus rimeporide-treated group, and † $p<0.05$ versus healthy control group. 2C, 3C and 4C mean 2-chamber, 3-chamber and 4-chamber, respectively.