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ORIGINAL CONTRIBUTION

Heart rate reduction and longevity in mice

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Abstract Heart rate correlates inversely with life span across all species, including humans. In patients with cardiovascular disease, higher heart rate is associated with increased mortality, and such patients benefit from pharmacological heart rate reduction. However, cause-and-effect relationships between heart rate and longevity, notably in healthy individuals, are not established. We therefore prospectively studied the effects of a life-long pharmacological heart rate reduction on longevity in mice. We hypothesized, that the total number of cardiac cycles is constant, and that a 15 % heart rate reduction might translate into a 15 % increase in life span. C57BL6/J mice received either placebo or ivabradine at a dose of 50 mg/kg/day in drinking water from 12 weeks to death. Heart rate and body weight were monitored. Autopsy was performed on all non-autolytic cadavers, and parenchymal organs were evaluated macroscopically. Ivabradine reduced heart rate by 14 % (median, interquartile range 12–15 %) throughout life, and median life span was increased by 6.2 % ($p = 0.01$). Body weight and macroscopic findings were not different between placebo and ivabradine. Life span was not increased to the same extent as heart rate was reduced, but nevertheless significantly prolonged by 6.2 %.

Keywords Heart rate · Ivabradine · Life span · Mice

Introduction

There is the popular belief, that god has given each individual a certain number of heart beats which determines their life span [20]. In fact, there is a close, inverse relation of heart rate to life span across all species, including humans [32, 43]. Heart rate is an independent risk factor for cardiovascular disease, notably myocardial ischemia and heart failure [4, 15, 22, 35]. Conversely, heart rate reduction provides benefit in terms of reduced mortality in patients with myocardial ischemia and/or heart failure [18, 22, 25, 45].

The existing studies, however, have not established a cause-and-effect relationship between heart rate and mortality. In mice, heart rate reduction by about 50 % with digoxin enhanced life span by 20 %, but also reduced body weight to the same extent [13]. Therefore, caloric restriction was not excluded as a potent confounder, which per se increases longevity [14, 34, 40].

Heart rate reduction without side effects is difficult, even under experimental conditions, and this is particularly true for a long-term study with mortality as endpoint.

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Electrical or surgical ablation of the sinus node induces trauma and entails the risk of arrhythmias, both of which might interfere with longevity. We therefore chose pharmacological heart rate reduction by ivabradine. Ivabradine acts preferentially on the hyperpolarization-activated cyclic nucleotide-gated channel (HCN) 4 in the sinus node [25] and has in contrast to beta-blockers, calcium antagonists or digoxin [1, 9, 16, 37] no effects on blood pressure or ventricular function, neither acutely in a pig model nor after repeated administration in mice or humans [2, 17, 27, 29]. Nevertheless, HCN channels are also localized in cardiac structures other than the sinus node, notably in ventricular tissue with hypertrophy and failure [5], and their blockade may result in non-specific actions of ivabradine [7, 38]. Even apart from the non-specific effects on HCN channels outside the sinus node, there may be other pleiotropic effects, which may not be related to HCN channels at all. Ivabradine reduced infarct size in pigs even when heart rate reduction was abrogated by atrial pacing, and in mice it reduced reactive oxygen species formation [17, 27].

We therefore prospectively studied a life-long pharmacological heart rate reduction by ivabradine on life span in mice. We used a dose of ivabradine in mice with the aim to reduce heart rate by 15 % and hypothesized an increase in life span by 15 %, along with the above popular belief.

Materials and methods

The experimental protocols were approved by the North Rhine Westphalia State Environment Agency (LUA NRW, Germany) (AZ: 8.87–50.10.37.09.104), and the investigation conforms with the Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23, revised 1996.

Male, 7–10 weeks old C57Bl6/J mice were purchased from Charles River (Charles River, Sulzfeld, Germany). Mice were kept singly in individually ventilated polypropylene cages under controlled conditions (12 h dark/12 h light cycle, humidity 50–60 %, temperature 21–23 °C) in one single room throughout the study. Access to the room was restricted to a small, stable group of researchers, animal keepers and the animal welfare officer. The mice had constant, free ad libitum access to commercial standard diet (R/M-H food pellets, ssniff, Soest, Germany) and drinking water. Food pellets were renewed and drinking water changed weekly.

Heart rate was recorded from an electrocardiogram (ECG) in non-anesthetized restrained mice. The signal was enhanced and digitized (Picoscope2203, Pico Technology Cambridgeshire, United Kingdom), and was recorded over 60–90 s. Heart rate was determined from RR intervals over 8 s in a stable steady state. The week-to-week variability of the individual heart rate was determined in 20 mice without and with ivabradine each over 12 weeks at the beginning of the study, and it was 5.2 (4.4–6.1) %.

Dose adjustment study

We aimed to reduce heart rate by about 15 %. The dose adjustment was started in 40 mice at 12 weeks of age with 10 mg/kg/day ivabradine added to the drinking water [19] (Fig. 1). In our hands, this dose reduced heart rate by only about 5.9 (4.4–7.3) %. Fluid intake was quantified daily over 2 weeks and was not different between placebo and ivabradine. A dose of 50 mg/kg/day reduced heart rate by 14.4 (12.4–16.9) % and also had no effect on fluid intake: 5.0 (4.6–5.8) ml/day with placebo vs. 5.1 (4.9–5.4) ml/day with ivabradine. The ivabradine plasma level was determined in 5 mice. Heparinized (500 I.E./500 µl blood) plasma was separated by centrifugation at 800*g* and 4 °C for 10 min and analyzed using liquid chromatography–mass spectrometry (LCMS/MS) by Servier (Suresnes, France). The ivabradine plasma level was 2.86 (median, interquartile range 2.34–6.98) or 8.69 ± 5.42 (mean ± SEM) ng/ml, respectively. Mice were killed after dose adjustment by cervical dislocation (Fig. 1).

Longevity study

An a priori power analysis for a 15 % increase in life span using the Wilcoxon–Mann–Whitney test (G*Power 3.1.7, Düsseldorf, Germany) was based on a prior study in untreated male C57Bl/6J mice which had lived for 676 ± 175 (mean ± SD) days [44] and resulted in a total sample size of 150 mice (statistical power 0.9, significance criterion 0.05, effect size 0.5, one-tail). To compensate for potential losses due to unexpected problems, the mouse number was increased to a total of 182 mice in our present study. Mice were randomized to ivabradine or placebo with blinded distribution of ID-cards; ivabradine treatment was started at 12 weeks of age and continued until death. No mice were added or lost during the study (Fig. 1).

Heart rate was measured in the same 20 mice each without and with ivabradine until their death. Dead mice were replaced by an age-matched mouse to maintain the number of mice with heart rate measurement constant. Body weight was monitored monthly in the same 10 mice each without and with ivabradine until they reached 600 days of age (Fig. 1).

Mice older than 600 days were weighed and their teeth status was monitored weekly to prevent death from starvation in old, senescent mice due to dedentition (Fig. 1). If necessary, teeth without counterbite were notched with a nipper without damaging the dental root according to veterinary instructions without sedation to prevent

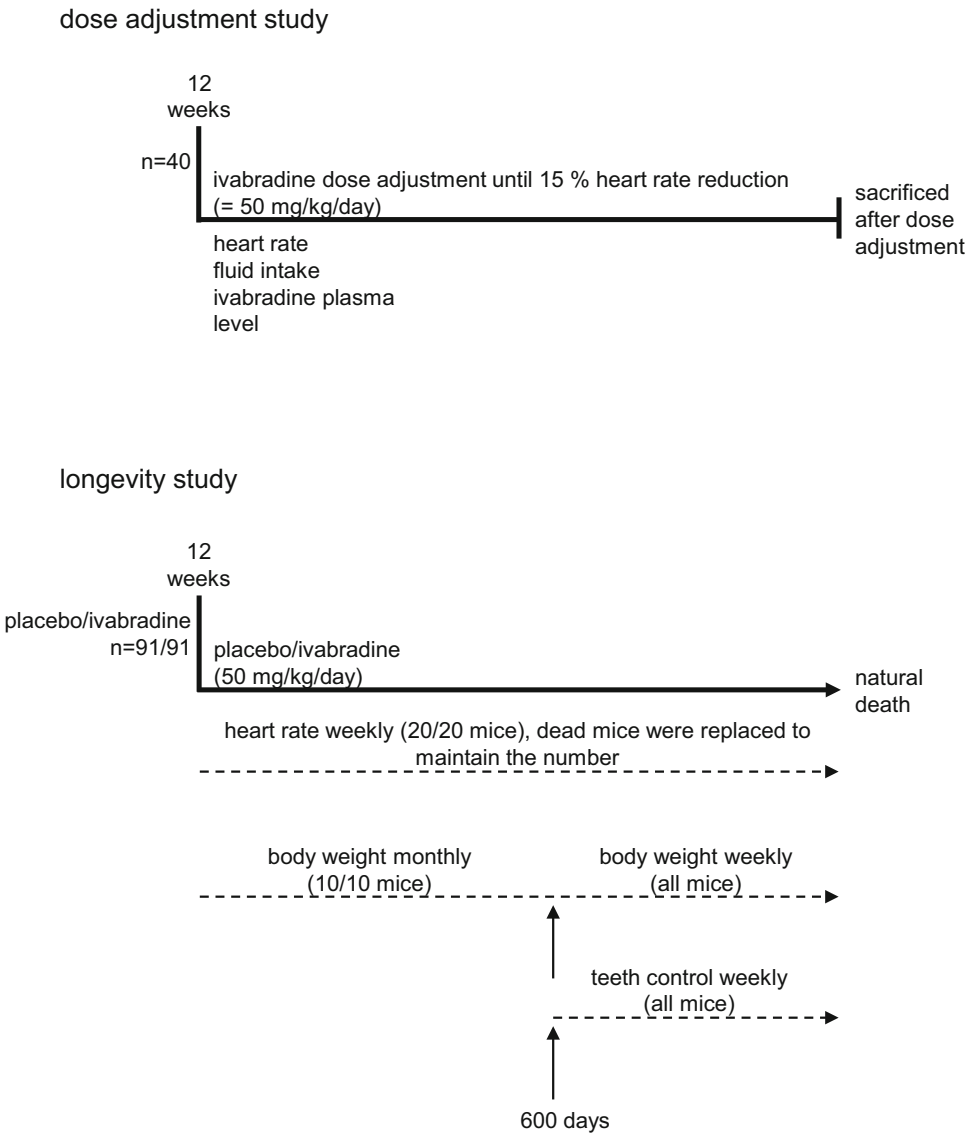

Fig. 1 Study design

overgrowing. During these regular inspections, we have observed in 3 mice (750 days of age and older) each without and with ivabradine a seizure, characterized by myoclonal limb contractions of a few minutes duration.

Mice with dedentition or a body weight reduction of more than 20 % or a body weight of less than 25 g were fed daily with food pellets soaked in drinking water with or without ivabradine; no differences between placebo and ivabradine were observed. All mice with signs of injury, illness or abnormal behavior were checked daily until natural death or until they fulfilled at least two of the following criteria: (1) paralysis and/or lethargy in spite of stimulation for more than 4 days; (2) body weight loss to less than 17 g without recovery within 48 h despite of tooth control and soaked food administration; (3) hypothermia for more than 24 h; (4) gasping for more than 4 h; (5) untreatable skin lesions of greater than 1 cm diameter; or (6) anal prolapse longer than 5 mm. The animal welfare officer of our institution was blinded to treatment and decided, whether a mouse fulfilled two or more of the above criteria and hence had to be killed. Seven mice each without and with ivabradine were killed by cervical dislocation (Table 1). Cadavers of non-autolytic mice (Fig. 6) were weighed and autopsied. Parenchymal organs were evaluated macroscopically, and hearts were weighed.

Histology of myocardial tissue

Cardiomyocyte cross-sectional area and myocardial fibrosis were analyzed in myocardial cross-sections of animals which had died early (between 570 and 740 days of age, $n = 6$ each without and with ivabradine) and late (between

Table 1 Reasons for euthanasia

	Placebo	Ivabradine
Euthanasia (*n*)	7	7
More than two of the following death criteria:		
Lethargy	4	4
Body weight loss	3	3
Hypothermia	3	4
Gasping	5	4
Untreatable skin lesions	3	2

980 and 1,170 days of age, $n = 10$ without, $n = 9$ with ivabradine). Hearts were excised during necropsy, formalin-fixed and paraffin-embedded. Cardiomyocyte cross-sectional area was quantified in hematoxylin- and eosin-stained tissue sections (18–22 cells in 5–7 fields of view of about 0.072 mm^2 each). Myocardial fibrosis was analyzed using Masson–Goldner trichrome-staining and quantified as percent of the field of view (3 fields of view of about 0.3 mm^2 each), as described previously [42].

Statistics

Data for survival time, heart rate and body weight failed the Kolmogorov–Smirnov test for normality and are presented as median with interquartile range (25–75 %). Unless stated otherwise, all statistical analyses were performed using SigmaStat (Systat software Inc., San Jose, USA). Kaplan–Meier survival functions were used to display the survival times without and with ivabradine and compared using the log rank test. In line with our hypothesis, the comparison was done in a one-tailed analysis [41]. Also, the median survival times were compared between placebo and ivabradine with the Wilcoxon-Mann–Whitney rank sum test in a one-tailed analysis. To address potential stress-related effects due to the restraint associated with heart rate measurements on longevity, median survival times were analyzed separately in those mice, which had and those, which had not undergone the heart rate measurement (placebo-restrained $n = 65$, ivabradine-restrained $n = 53$, placebo-non-restrained $n = 26$, ivabradine-non-restrained $n = 38$) and compared with a two-way analysis of variance. To address a potential relationship between heart rate and life span on an individual basis, mean heart rate was calculated for each individual mouse over all available heart rate measurements (more than 3 data points) and correlated to its individual life span using an analysis of covariance (SAS version 9.2, Cary, USA). Heart rate was analyzed as area under the curve (AUC) throughout life including replaced mice and also compared in a one-tailed analysis with the Wilcoxon-Mann–Whitney rank sum test to test only for the heart rate reduction with ivabradine. Body weight was analyzed in two parts, before 600 days for 10 mice without and with ivabradine and after 600 days for all mice. Body weight was analyzed as AUC from 60 to 600 days and also from 600 days until death (standardized by dividing by the time until death) and 95 % confidence intervals [36] were compared (StatXact version 6.0, Cytel, Cambridge, USA). Heart weight, body weight and the quotient of heart and body weight were analyzed with a two-tailed Wilcoxon-Mann–Whitney rank sum test. Macroscopic findings are given in percent of the number of non-autolytic cadavers. Data for cardiomyocyte cross-sectional area and myocardial fibrosis passed the Kolmogorov–Smirnov test for normality. Therefore, these data are expressed as mean ± standard error of the mean (SEM) and analyzed by a two-way analysis of variance followed by Bonferroni post hoc test. $p < 0.025$ for one-tailed and $p < 0.05$ for two-tailed analysis were considered significant.

Results

Heart rate was reduced by 14 (12–15) % with ivabradine vs. placebo [583 (559–607) to 680 (639–698) min^{-1}, $p < 0.001$] (Fig. 2a). Kaplan–Meier survival functions (Fig. 2b) displayed the increased survival time of mice with ivabradine ($p = 0.023$). Life-long heart rate reduction increased median life span from 906 (785–1,015) to 962 (864–1,066) days, $p = 0.01$, i.e., by 6.2 % (Fig. 2b, insert). Median life span was enhanced in restrained mice, both without and with ivabradine, over those without restraint ($p < 0.001$) (Fig. 3). Ivabradine still significantly increased median life span over that with placebo in restrained mice and by trend also in non-restrained mice (Fig. 3). The estimated median number of cardiac cycles per life (i.e., median heart rate x median life span) was 8.5×10^8 for mice without and 7.9×10^8 for mice with ivabradine. An inverse relationship existed between individual heart rate and life span in all tested mice ($p = 0.046$), and no significant interactions or differences between mice without and with ivabradine were detected (Fig. 4). Body weight was not different between placebo and ivabradine (Fig. 5). Also, macroscopic findings were not different between placebo and ivabradine (Fig. 6). Cardiomyocyte cross-sectional area increased with aging, however, this effect was independent of treatment (without and with ivabradine) (Table 2). Myocardial fibrosis was neither different between mice which died early and late nor did it depend on treatment (without and with ivabradine) (Table 2).

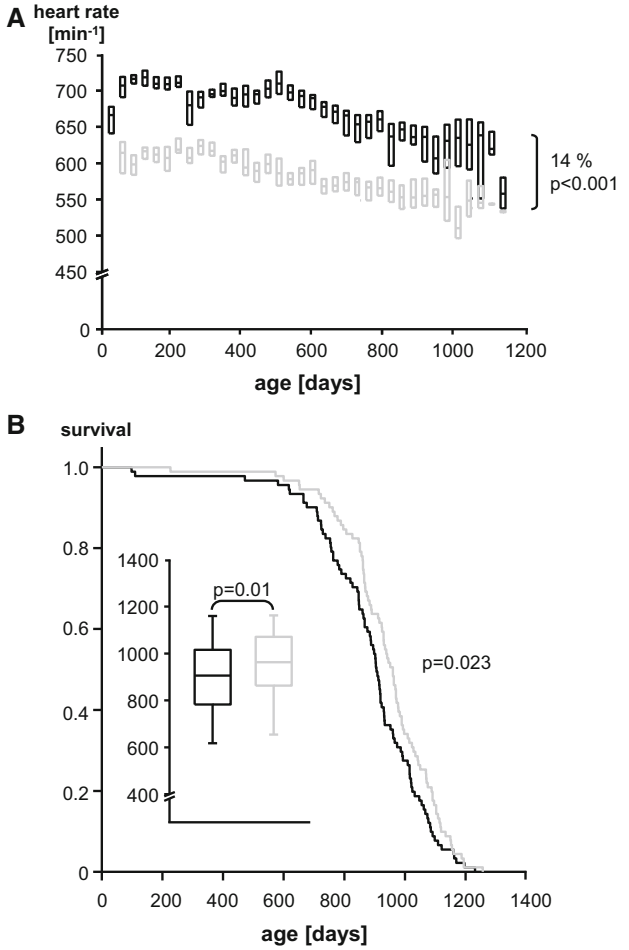

Fig. 2 Heart rate (**a**), Kaplan–Meier survival functions (**b**) and median life span (*insert*) of placebo (*black*) and ivabradine (*grey*) mice. Heart rate was reduced by ivabradine (**a**), and life span was increased (**b**, *insert*)

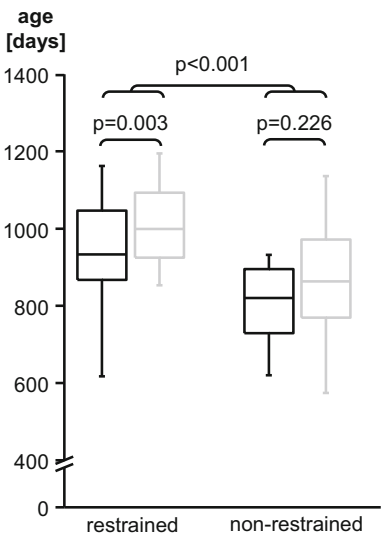

Fig. 3 Median life span of placebo (*black*) and ivabradine (*grey*) restrained and non-restrained mice

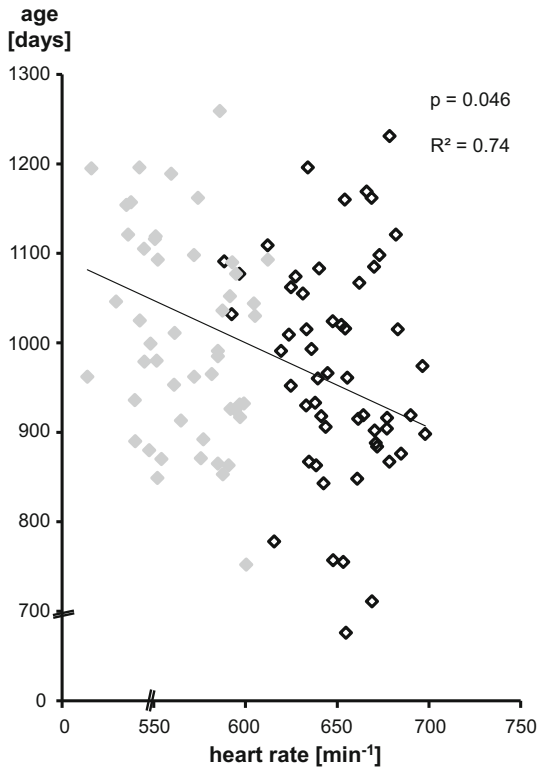

Fig. 4 Relationship between heart rate and life span. The mean heart rate was plotted against the individual life span; placebo (*black*) and ivabradine (*grey*)

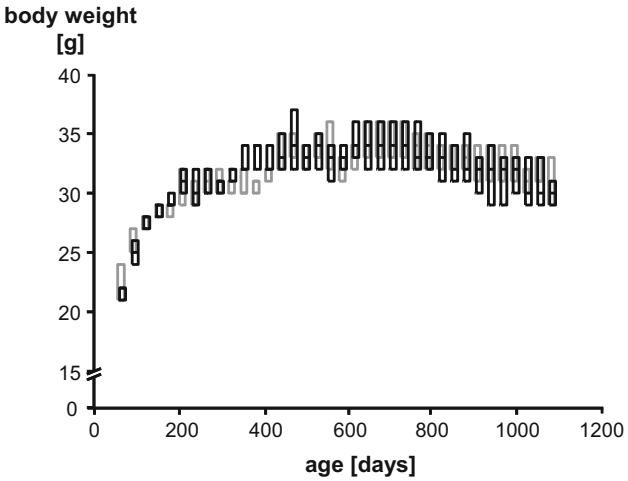

Fig. 5 Body weight was not different between placebo (*black*) and ivabradine (*grey*)

Discussion

In the present study, life-long heart rate reduction by 14 (12–15) % with ivabradine increased life span by 6.2 %. Thus, the popular belief of a fixed number of cardiac cycles for a given life is not correct. Nevertheless, when translated

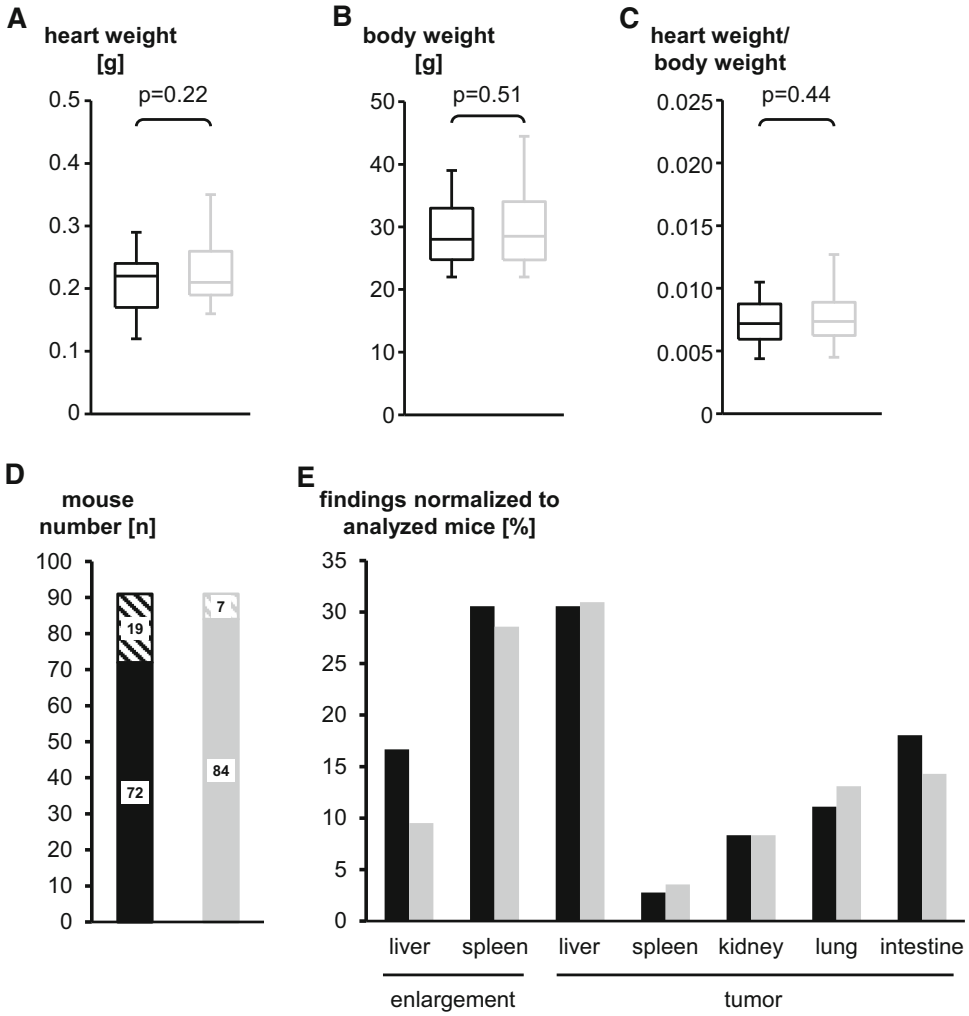

Fig. 6 Heart weight (**a**), body weight (**b**) and heart weight/body weight (**c**) were not different between placebo (*black*) and ivabradine (*grey*). The number of autolytic mice (*stripes*) and non-autolytic with placebo (*black*) and ivabradine (*grey*) is shown in (**d**); macroscopic findings were normalized to the number of non-autolytic mice and were not significantly different between placebo (*black*) and ivabradine (*grey*)

Table 2 Histological analysis of myocardial tissue

	Cardiomyocyte cross-sectional area (μm^2)	Myocardial fibrosis (%)
Died early (between 570 and 740 days of age)		
Placebo	383 ± 50	0.7 ± 0.2
Ivabradine	312 ± 22	1.0 ± 0.2
Died late (between 980 and 1,170 days of age)		
Placebo	411 ± 19*	1.1 ± 0.1
Ivabradine	433 ± 10*	0.9 ± 0.1

* $p < 0.05$ compared to early died

to humans, a 6.2 % increase in life span equates 5 years for a person at 80 years of age. Only one study so far has examined the effect of life-long pharmacological heart rate reduction on the life span in healthy mice: digoxin reduced heart rate in mice by about 50 % and increased their life span by 20 % [13]. However, body weight was also reduced by about 20 %, and a significant role for caloric restriction in the observed increase in longevity could not be ruled out [13]. Of note, caloric restriction is the most powerful life span enhancing strategy known yet [14, 34, 40]. Therefore, we have used the relatively selective bradycardic agent ivabradine [25] to reduce heart rate, and indeed body weight was not affected.

In the present study, heart rate decreased with aging, confirming reports from prior longitudinal studies in mice [33] and humans [46]. Also, in a cross-sectional study of aged humans, heart rate was lower than in younger individuals [43]. However, in such cross-sectional study it remains unclear whether the reduced heart rate reflects an age-dependent decline or is the result of a selection bias.

Progeria, a syndrome of accelerated aging has surprisingly not been associated with increased heart rate, not in humans [24] and not in a mouse model of progeria [8], although patients with progeria often die from myocardial infarction or stroke [23]. In contrast, in humans with obesity and metabolic syndrome heart rate is increased, possibly reflecting higher sympathetic activity [31, 39]. The metabolic syndrome is also associated with a reduced life span [21]. The causality of these associations is unclear. Therefore, it would be interesting for future studies to analyze the effect of life-long heart rate reduction with ivabradine in a mouse model of obesity/metabolic syndrome [28, 30].

The restraining process associated with the heart rate measurement certainly induced stress and increased heart rate. However, this is almost the same phenomenon when a patient has measurements of heart rate and blood pressure taken in the doctoŕs office, and such "white coat" effect does not detract from the diagnostic and prognostic values of such measurements. In the present study, the restraint associated with the heart rate measurement improved longevity. Ivabradine still increased longevity in these mice. Possibly, the restraint-related repeated stress exerted a conditioning effect, such as seen with regular exercise [47]. The greatest survival difference between mice without and with ivabradine existed already relatively early on (500–600 days) in the Kaplan–Meier survival function. We could not determine the causes of death, therefore distinction of early cardiovascular vs. later cancer deaths would be highly speculative. The observed increase of cardiomyocyte cross-sectional area with age is in line with prior reports on aged C57Bl6/J mice [10]. Also, in C57Bl6/J mice, fibrosis increases over the first year of life, but the lack of further increase in fibrosis with further aging in our study is in line with prior reports [11, 12]. The life-long heart rate reduction with ivabradine did neither impact on cardiomyocyte cross-sectional area nor on myocardial fibrosis.

We could not determine whether the improved longevity in our present study was secondary to heart rate reduction or to pleiotropic effects of ivabradine per se [26]. Pleiotropic effects of ivabradine were observed in anesthetized pigs, where ivabradine had effects partially independent of heart rate reduction [27]. Ivabradine reduced the formation of reactive oxygen species [17], but it was not clear in that study using atherosclerotic mice whether or not the reduced reactive oxygen formation from NADPH oxidase was secondary to heart rate reduction or else. Reduced reactive oxygen species formation, either as a consequence of heart rate reduction or a direct drug action of ivabradine, would be mechanistically consistent with improved longevity [3].

Study limitations

In the present study, we cannot attribute a causal role to heart rate reduction in providing greater longevity, since we cannot separate heart rate-dependent from non-heart rate-dependent effects of ivabradine. Nevertheless, the good correlation of individual heart rate and longevity suggests that heart rate reduction has a role in longevity.

Heart rate was recorded from ECG in restrained, non-anesthetized mice. Telemetry would have been an alternative method, but due to the traumatic nature of its implantation and the risk of inflammation, it might have interfered with longevity.

We used mice for the present study since their life span of about 3 years was technically feasible for our purpose. However, mice die mostly from cancer [6, 44] and not from cardiovascular disease, where the benefit from heart rate reduction may be more obvious.

In conclusion, selective heart rate reduction by ivabradine increased longevity by 6.2 %, which in humans translates to an additional 5 years for a person of 80 years.

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Conflict of interest GH serves as a consultant to Servier.

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