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Short Communication

Ivabradine curbs isoproterenol-induced kidney fibrosis

Tomas Baka[1,*], Peter Stanko[1,*], Kristina Repova[1,*], Silvia Aziriova[1,2], Kristina Krajcirovicova[1], Andrej Barta[3], Stefan Zorad[4] and Fedor Simko[1,4,5]

[1] *Institute of Pathophysiology, Faculty of Medicine, Comenius University, Bratislava, Slovakia*
[2] *Department of Psychology, Faculty of Arts, Comenius University, Bratislava, Slovakia*
[3] *Institute of Normal and Pathological Physiology, Centre of Experimental Medicine, Slovak Academy of Sciences, Bratislava, Slovakia*
[4] *Institute of Experimental Endocrinology, Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovakia*
[5] *3rd Department of Internal Medicine, Faculty of Medicine, Comenius University, Bratislava, Slovakia*

Abstract. This study investigated whether chronic isoproterenol administration could induce kidney alterations and whether ivabradine, a heart rate (HR)-reducing substance exerting cardiovascular protection, is able to attenuate potential kidney damage. Twenty-eight Wistar rats were divided into non-diseased controls, rats treated with ivabradine, rats treated with isoproterenol, and rats treated with isoproterenol plus ivabradine. Six weeks of isoproterenol administration was associated with decreased systolic blood pressure (SBP) (by 25%) and glomerular, tubulointerstitial and vascular/perivascular fibrosis due to enhanced type I collagen volume (7-, 8-, and 4-fold, respectively). Ivabradine reduced HR (by 15%), partly prevented SBP decline (by 10%) and site-specifically mitigated kidney fibrosis by decreasing type I collagen volume in all three sites investigated (by 69, 58, and 67%, respectively) and the ratio of type I collagen-to-type III collagen in glomerular and vascular/perivascular sites (by 79 and 73%, respectively). We conclude that ivabradine exerts protection against kidney remodelling in isoproterenol-induced kidney damage.

Key words: Ivabradine — Isoproterenol — Kidney fibrosis — Chronic kidney disease — Cardiorenal syndrome

Heart failure (HF) and chronic kidney disease (CKD) are frequently concurrent, as they share common risk factors, including hypertension and type 2 diabetes. Indeed, the prevalence of HF in CKD patients, or *vice versa* CKD in HF patients, is approximately four-to-five times the prevalence of HF or CKD in the general population (McCullough et al. 2013; House et al. 2019; Romero-González et al. 2020; Jankowski et al. 2021). Moreover, the coexistence of HF and CKD substantially precipitates adverse cardiovascular events and remarkably worsens prognosis (Hillege et al. 2006; McCullough et al. 2013). This is potentially determined by common pathomechanisms of HF and CKD, including neurohumoral activation, free radical and inflammatory cytokine burden, or increased expression of profibrotic factors, which collectively underlie the pathological remodelling of both the heart and the kidneys (McCullough et al. 2013; House et al. 2019; Romero-González et al. 2020; Jankowski et al. 2021). Along with blood pressure, heart rate (HR) is an easily accessible haemodynamic factor used in cardiovascular risk assessment, while elevated HR was shown to be associated with adverse cardiovascular prognosis in both HF and CKD (Cice et al. 2008; Voors et al. 2014; Simko et al. 2016; Baka and Simko 2018).

* These authors contributed equally to this work.
Correspondence to: Fedor Simko, Institute of Pathophysiology, Faculty of Medicine, Comenius University, Sasinkova 4, 811 08 Bratislava, Slovakia
E-mail: fedor.simko@fmed.uniba.sk

Ivabradine reduces HR by selective I_f current inhibition in the sinoatrial node. Besides slowing HR down, ivabradine has been shown to exert a number of pleiotropic effects of potential cardiovascular benefit, such as anti-inflammatory and antioxidant actions or mitigation of the cytokine release and neurohumoral activation (Aziriova et al. 2016; Baka and Simko 2019a, 2019b; Simko and Baka 2021). In the SHIFT study, ivabradine reduced the composite primary endpoint of cardiovascular death and hospitalizations due to worsening HF in patients with chronic systolic HF and elevated HR (Swedberg et al. 2010). In addition, in a SHIFT sub-analysis encompassing patients with CKD, ivabradine also effectively reduced the composite primary endpoint (Voors et al. 2014). Moreover, recently we have shown ivabradine to exert antiremodelling and antifibrotic effects on an isoproterenol-damaged heart in rats (Simko et al. 2021) and hypertensive kidneys in a rat model of N^G-nitro-l-arginine methyl ester (L-NAME)-induced hypertension (Stanko et al. 2020). Both HR-reduction and pleiotropic effects were considered to participate in cardiovascular protection by ivabradine. It seems justified to suppose that ivabradine may not only protect the heart but also curb kidney damage in various cardiovascular pathologies.

Isoproterenol-induced myocardial injury is an established and frequently used HF model (Nichtova et al. 2012; Simko et al. 2014, 2021), but the data on isoproterenol's effect on kidneys are insufficient. Here, we sought to determine whether isoproterenol induces fibrotic rebuilding of the kidneys and whether ivabradine is able to protect against kidney remodelling.

Twenty-eight 12-week-old weight-matched male Wistar rats (Department of Toxicology and Laboratory Animals Breeding, Slovak Academy of Sciences, Dobra Voda, Slovakia) were randomly divided into four groups ($n = 7$ *per* group) and treated for six weeks as follows: control (C; untreated), ivabradine (Iva; 10 mg/kg/day orally; Servier, Suresnes, France), isoproterenol (Iso; 5 mg/kg/day intraperitoneally; Sigma-Aldrich, Taufkirchen, Germany), and isoproterenol plus ivabradine (Iso+Iva; corresponding doses). Animals in the C and Iva groups were intraperitoneally injected with a saline vehicle. Ivabradine was dissolved in drinking water and its concentration was adjusted to daily water consumption to ensure the correct dosage. Animals were housed in individual cages, maintained under standard laboratory conditions (12:12-hour light-dark cycle at 22–24°C temperature and 45–65% humidity) and fed a regular pellet diet *ad libitum*. Systolic blood pressure (SBP) and HR were measured once a week in each animal by non-invasive tail-cuff plethysmography (Hugo-Sachs Elektronik, Freiburg, Germany). After six weeks of treatment, the rats were euthanized by isoflurane inhalation and the left kidneys were harvested for subsequent histopathological analysis. The kidney samples were fixed in 4% formaldehyde for 24 h, embedded in paraffin and cut into 5 μm-thick sections. Thereafter, the sections were deparaffinised, rehydrated and stained with picrosirius red (PSR) for a quantitative analysis of kidney fibrosis. Photomicrographs were taken through a NIKON Eclipse Ti C2+ microscope (NIKON, Tokyo, Japan) with transmitted and polarized light and subsequently analysed using ImageJ version 1.52p for Windows (National Institutes of Health, Bethesda, MD, USA) (Pechanova et al. 2006; Hrenák et al. 2013).

In order to obtain a quantitative analysis of kidney fibrosis, PSR-stained sections were analysed using polarized light microscopy at 100× magnification and ImageJ as described by our laboratory and others (Seccia et al. 2008; Stanko et al. 2020; Repova et al. 2022). Briefly, the birefringence shift by PSR visualizes the thick type I collagen (Col-I) in red-orange shades and the thin type III collagen (Col-III) in green-yellow shades. The volume of Col-I or Col-III was determined as the percentage of red-orange or green-yellow shaded area in a particular region of interest (ROI) by setting the appropriate "hue" thresholds of the colour spectrum in ImageJ. Therefore, results represent the percent of Col-I or Col-III-associated colour, and thus the volume of Col-I or Col-III, in particular ROI. Three sets of ROIs were determined to assess: (i) glomerular fibrosis by placing 50 ROIs *per* section of 50×50 μm in the intraglomerular space; i.e., 350 intraglomerular ROIs were investigated *per* group; (ii) tubulointerstitial fibrosis by placing 50 ROIs *per* section of 192×72 μm in the interstitial cortex, including no glomeruli or vessels; i.e., 350 tubulointerstitial ROIs were investigated *per* group; and (iii) vascular/perivascular fibrosis by selecting 5 ROIs *per* section, tight-cropping a cross-sectionally captured artery with a diameter between 50 and 100 μm; i.e., 35 vascular/perivascular ROIs were investigated *per* group.

All histopathological analyses were performed by an experienced investigator blinded to the group identity. The study was conducted in conformance with 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). The protocol was approved by the ethics committee at the Institute of Pathophysiology, Faculty of Medicine, Comenius University, Bratislava, Slovakia.

The results are presented as the mean ± SEM. The one-way two-tailed analysis of variance (ANOVA) followed by a Bonferroni's multiple comparisons test was used for statistical analysis. Statistical significance was defined as $p < 0.05$. The statistical analysis was conducted using GraphPad Prism 9 for Windows (GraphPad Software, La Jolla, CA, USA).

SBP averaged over the course of the experiment was 126.10 ± 0.73 mmHg in controls and isoproterenol decreased ($p < 0.0001$) it to 94.33 ± 2.45 mmHg (by 25%). Ivabradine decreased ($p < 0.0001$) the average SBP to 113.98 ± 0.99 mmHg (by 10%) in the control group and increased ($p <$

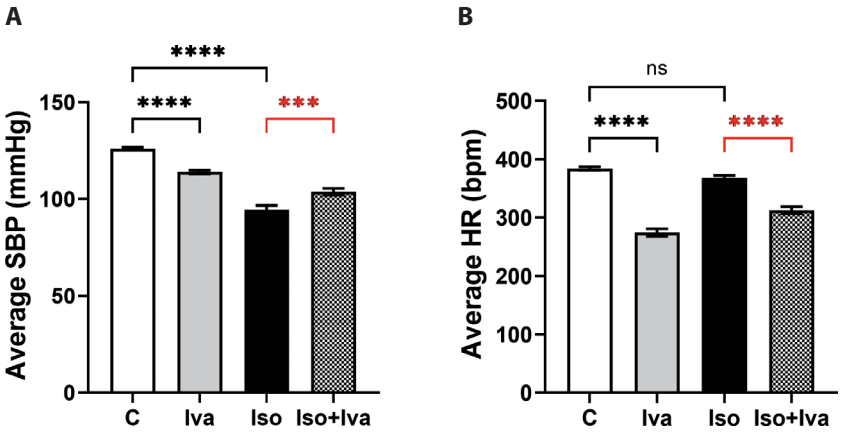

Figure 1. Effect of ivabradine on average systolic blood pressure (SBP; **A**) and average heart rate (HR; **B**) in a rat model of isoproterenol-induced kidney injury after six weeks of treatment. $n = 7$ animals *per* group. One-way two-tailed ANOVA followed by Bonferroni's multiple comparisons test; ns, non-significant; *** $p < 0.001$; **** $p < 0.0001$. Animal groups: C, controls; Iva, rats treated with ivabradine (10 mg/kg/day orally); Iso, rats treated with isoproterenol (5 mg/kg/day ip); Iso+Iva, rats treated with isoproterenol (5 mg/kg/day ip) and ivabradine (10 mg/kg/day orally).

0.001) it to 103.84 ± 1.71 mmHg (by 10%) in the isoproterenol group (Fig. 1A). HR averaged over the course of the experiment was 384.27 ± 2.84 bpm in controls, and isoproterenol had no significant effect at the time of HR measurement (368.38 ± 3.74 bpm). Ivabradine decreased ($p < 0.0001$) the average HR in both the control and isoproterenol group to 274.16 ± 6.51 bpm (by 29%) and 312.74 ± 5.83 bpm (by 15%), respectively (Fig. 1B).

Representative images of PSR-stained sections using transmitted and polarized light microscopy are depicted in Figure 2.

In the intraglomerular ROIs, the volume of Col-I and Col-III in the controls were 0.99 ± 0.14% and 4.22 ± 0.81%, respectively, and their ratio was 0.26 ± 0.03. Isoproterenol increased ($p < 0.0001$) the volume of Col-I by 742% and had no significant effect on the volume of Col-III, thus increasing ($p < 0.0001$) the Col-I:Col-III ratio by 563%. In the isoproterenol group, ivabradine decreased ($p < 0.0001$) the volume of Col-I by 69% without affecting the volume of Col-III, thus decreasing ($p < 0.0001$) the Col-I:Col-III ratio by 79% (Fig. 3A).

In the tubulointerstitial ROIs, the volume of Col-I and Col-III in the controls were 0.45 ± 0.10% and 1.64 ± 0.42%, respectively, and their ratio was 0.31 ± 0.04. Isoproterenol increased the volume of both Col-I and Col-III by 809% ($p < 0.0001$) and 201% ($p < 0.01$), respectively, and it increased ($p < 0.01$) the Col-I:Col-III ratio by 234%. In the isoproterenol group, ivabradine decreased ($p < 0.01$) the volume of Col-I by 58% without affecting the volume of Col-III or the Col-I:Col-III ratio (Fig. 3B).

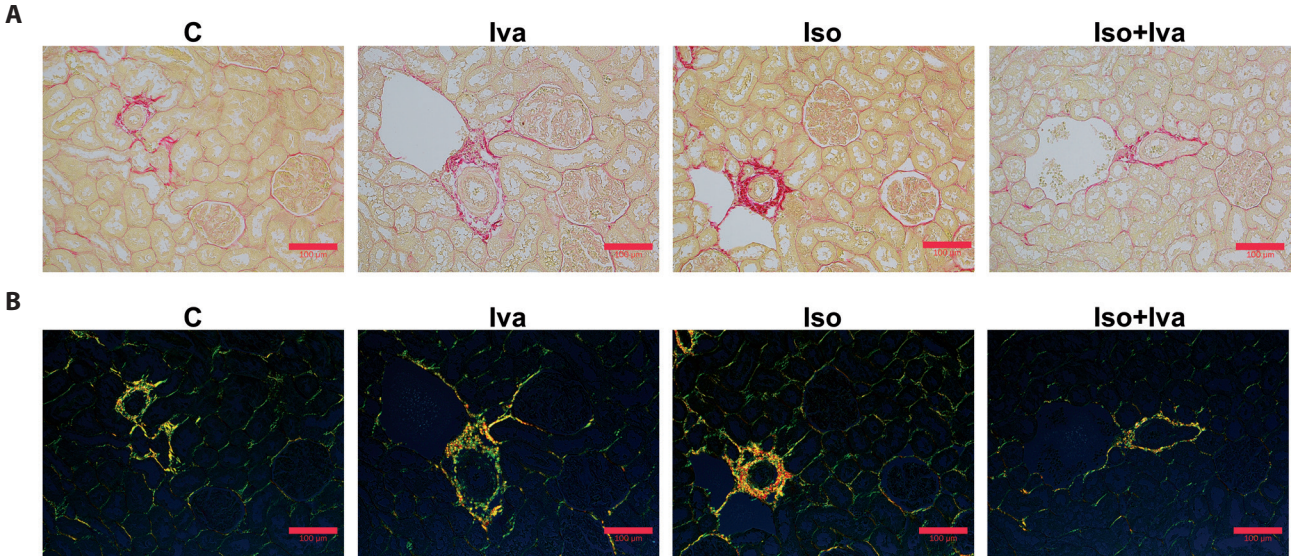

Figure 2. Representative images of picrosirius red (PSR)-stained sections at 100× magnification using transmitted (**A**) and polarized (**B**) light microscopy. For more abbreviations, see Figure 1.

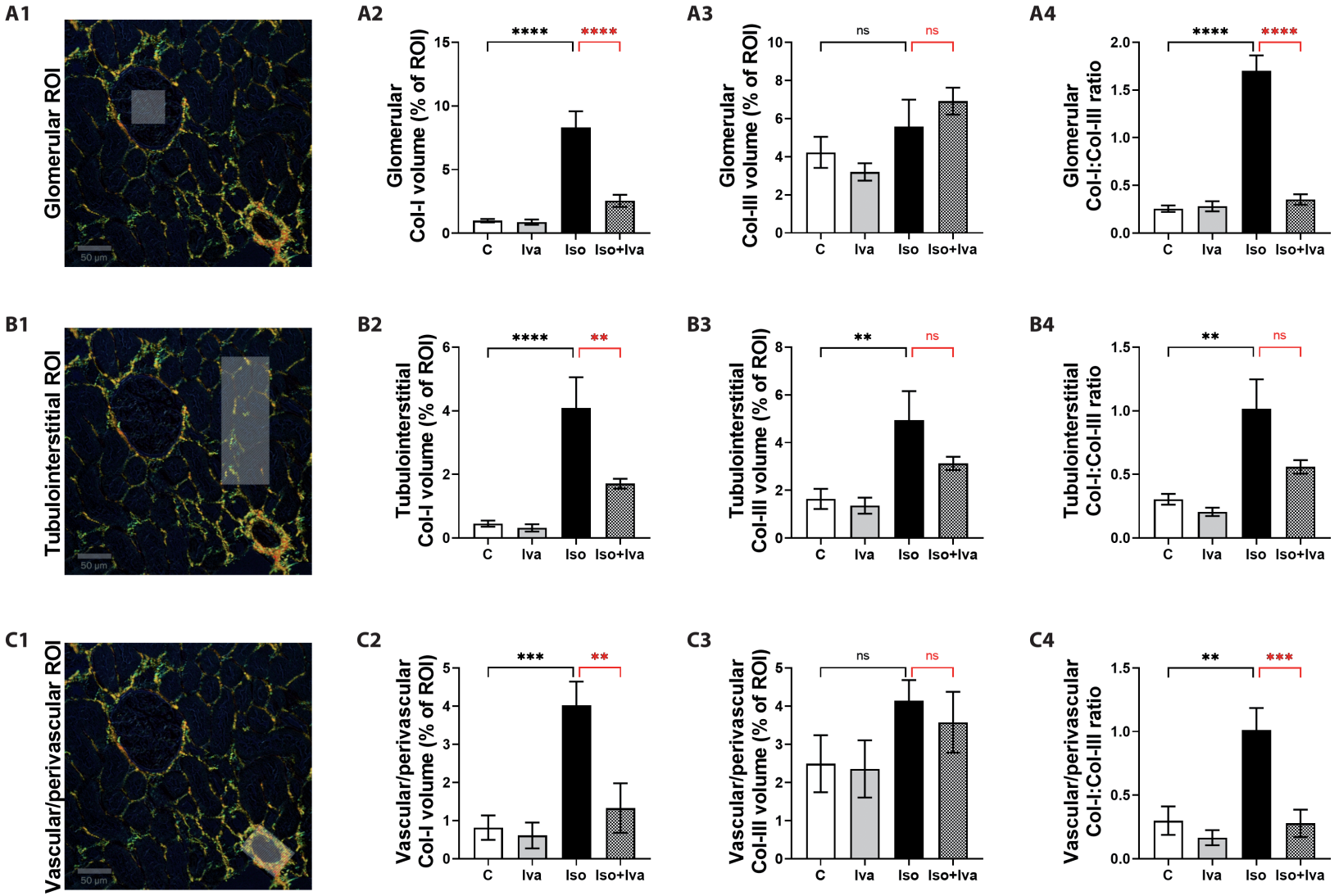

Figure 3. Effect of ivabradine on kidney fibrosis detailed as glomerular (**A**), tubulointerstitial (**B**), and vascular/perivascular fibrosis (**C**) in a rat model of isoproterenol-induced kidney injury after six weeks of treatment. The first column shows the glomerular (**A1**), tubulointerstitial (**B1**), and vascular/perivascular (**C1**) ROI in a representative image. The volume of type I collagen (**A2, B2** and **C2**), or type III collagen (**A3, B3** and **C3**) and their ratio (**A4, B4** and **C4**) in a particular ROI are depicted in the second, third and fourth column, respectively. Picrosirius red (PSR)-stained sections at 100× magnification using polarized light microscopy. $n = 7$ animals *per* group. One-way two-tailed ANOVA followed by Bonferroni's multiple comparisons test; ns, non-significant; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Col-I, type I collagen; Col-III, type III collagen; ROI, region of interest depicted as shaded rectangle. For more abbreviations, see Figure 1.

In the vascular/perivascular ROIs, the volume of Col-I and Col-III in the controls were 0.81 ± 0.32% and 2.49 ± 0.75%, respectively, and their ratio was 0.30 ± 0.11. Isoproterenol increased ($p < 0.001$) the volume of Col-I by 394% and had no significant effect on the volume of Col-III, thus increasing ($p < 0.01$) the Col-I:Col-III ratio by 239%. In the isoproterenol group, ivabradine decreased ($p < 0.01$) the volume of Col-I by 67% without affecting the volume of Col-III, thus decreasing ($p < 0.001$) the Col-I:Col-III ratio by 73% (Fig. 3C).

Treatment with isoproterenol was associated with decreased SBP and profound kidney fibrosis. Ivabradine reduced HR, partly prevented SBP decline and site-specifically mitigated kidney fibrosis by decreasing the Col-I volume in all three sites investigated (glomerular, tubulointerstitial and vascular/perivascular) and the Col-I:Col-III ratio in glomerular and vascular/perivascular ROIs. To the best of our knowledge, this is the first study analysing isoproterenol-induced kidney fibrosis and ivabradine's renoprotective effect site-specifically.

The rat model of isoproterenol-induced heart and kidney injury mimics the features of cardiorenal syndrome in terms of neurohumoral activation and inflammation, vascular senescence and remodelling, and heart and kidney dysfunction, remodelling and fibrosis (Liu et al. 2015; Adamcova et al. 2019; Hasan et al. 2020; El-Shaer et al. 2021). Ivabradine was recently shown to improve cardiac function and haemodynamics in acute isoproterenol-induced heart injury (Ali et al. 2020) and to mitigate the left-ventricular hypertrophy, fibrosis and SBP decline associated with improved survival in chronic isoproterenol-induced heart injury (Simko et al. 2021). Although data on the effect of ivabradine on isoproterenol-induced kidney injury are not available in the literature, we have previously shown ivabradine to ameliorate kidney damage and fibrosis in a site-specific manner in a rat model of L-NAME-induced hypertension (Stanko et al. 2020). Col-I and Col-III, important components of the extracellular matrix, were found to be progressively deposited in glomeruli, the tubulointerstitium and vasculature during kidney fibrosis (Bülow and Boor 2019). As Col-I is stiff and Col-III is rather elastic, the Col-I:Col-III ratio determines the biomechanical properties of the remodelled tissue (Asgari et al. 2017).

In this study, isoproterenol-induced kidney fibrosis was associated with an elevated Col-I:Col-III ratio at all three investigated sites (glomerular, tubuloinsterstitial, and vascular/perivascular); ivabradine's antifibrotic effect was shown to be site-specific, as it reduced the Col-I:Col-III ratio in the glomeruli and vasculature. This finding corroborates the results of our previous study on ivabradine's effect on kidney fibrosis in L-NAME-hypertension (Stanko et al. 2020). Of note, ivabradine's antifibrotic effect on vasculature was already shown in the aortas of diabetic mice (Reil et al. 2013) and the coronary arteries of rats with myocardial infarction (Dedkov et al. 2007). The reduction of the Col-I:Col-III ratio by ivabradine found in this study implies increased compliance of glomerular capillaries and arterioles, potentially leading to decreased renal vascular resistance with better haemodynamics and renal blood flow autoregulation maintaining an appropriate glomerular filtration rate (Stanko et al. 2020; Simko and Baka 2021). Moreover, ivabradine was shown to mitigate low shear stress-induced endothelial inflammation and injury, as it decreased the expression of IL-6 and VCAM-1 and increased the expression of endothelial nitric oxide synthase (eNOS) (Li et al. 2016), and to improve endothelial function in ApoE knockout mice associated with decreased NADPH oxidase activity, the prevention of eNOS uncoupling and attenuation of angiotensin II signalling (Kröller-Schön et al. 2011).

The mechanisms underlying ivabradine's antifibrotic renal protection are only hypothetical. They may reside in the prevention of blood pressure decline, supposedly indicating improved left ventricular function (Simko et al. 2021) and renal perfusion (Stanko et al. 2020), in mitigating angiotensin II's and aldosterone's profibrotic impact (Busseuil et al. 2010; Kröller-Schön et al. 2011; Simko et al. 2018; Simko and Baka 2021), or in ivabradine's direct anti-oxidative and anti-inflammatory effects (Kröller-Schön et al. 2011; Custodis et al. 2012; Li et al. 2016; Simko and Baka 2021). Collectively, based on the findings of this study and extrapolating data from other studies, ivabradine seems to exert not only cardiac but also renal protection in a model of isoproterenol-induced cardiovascular damage. It therefore seems reasonable to assume that chronic isoproterenol-induced remodelling of the heart and kidneys may be considered a suitable model for testing cardiorenal protectives.

In conclusion, our results suggest that ivabradine may be protective against isoproterenol-induced fibrotic rebuilding of the kidneys.

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Conflict of interest. None.

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