

Sex-Linked Differences in Cardiac Atrophy After Mechanical Unloading Induced by Heterotopic Heart Transplantation

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Summary

No information is available about sex-related differences in unloading-induced cardiac atrophy. We aimed to compare the course of unloading-induced cardiac atrophy in intact (without gonadectomy) male and female rats, and in animals after gonadectomy, to obtain insight into the influence of sex hormones on this process. Heterotopic heart transplantation (HT_x) was used as a model for heart unloading. Cardiac atrophy was assessed as the weight ratio of heterotopically transplanted heart weight (HW) to the native HW on days 7 and 14 after HT_x in intact male and female rats. In separate experimental groups, gonadectomy was performed in male and female recipient animals 28 days before HT_x and the course of cardiac atrophy was again evaluated on days 7 and 14 after HT_x. In intact male rats, HT_x resulted in significantly greater decreases in whole HW when compared to intact female rats. The dynamics of the left ventricle (LV) and right ventricle (RV) atrophy after HT_x was quite similar to that of whole hearts. Gonadectomy did not have any significant effect on the decreases in whole HW, LV, and RV weights, with similar results in male and female rats. Our results show that the development of unloading-induced cardiac atrophy is substantially reduced in female rats when compared to male rats. Since gonadectomy did not alter the course of cardiac atrophy after HT_x, similarly in both male and female rats, we conclude that sex-linked differences in the development of unloading-induced cardiac atrophy are not caused by the activity of sex hormones.

Key words

Cardiac atrophy • Sex differences • Gonadectomy • Heterotopic heart transplantation • Mechanical heart unloading

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Introduction

Heart failure (HF) is a global problem with at least 38 million patients worldwide, with the number of new HF patients estimated to increase by 50 % per year [1,2]. Even though 59.5 % of all annual deaths of HF patients are women, this risk factor remains underestimated, not only in the general public but also among female healthcare professionals, where the risk of death connected with breast cancer is thought to be higher than in heart disease [2]. The original epidemiological study indicating that women with HF exhibit a lower risk of mortality [3] was challenged: more recent studies showed that in the cohort of HF patients with reduced ejection fraction, women displayed a higher mortality rate [4-6]: moreover, women with HF reported a worse quality of life [2,5,6]. Fortunately, the awareness of critically important sex-related differences in the risk and

course of HF [7-10] is growing, and it is accepted that there is an urgent need for studies elucidating biological mechanisms underlying sex-related differences in the pathophysiology of HF and in responses to new therapeutic measures [7,11,12].

In line with the growing HF epidemic (in both sexes), the number of patients with end-stage HF is on the rise [1,2]. Heart transplantation (HT_x) is the best treatment approach for such patients, yet the value of HT_x as a treatment option is limited mainly due to a scarcity of quality organ donors, therefore the implantation of left ventricle assist devices (LVAD) has emerged as an alternative treatment for patients with end-stage HF, with clinical use ever increasing [13-15].

At present, long term durable LVAD implantation is indicated in a wide range of conditions, most commonly as a “bridge to transplantation” in patients with end-stage HF awaiting HT_x, as “destination therapy” in patients with end-stage HF that are not eligible for HT_x, and as a “bridge to recovery”, in patients with end-stage HF in whom LVAD either alone or in combination with other therapy may be effective in reversing maladaptive left ventricle (LV) remodeling: this process is referred to as “reverse remodeling” and should improve cardiac function leading to the weaning from LVAD treatment [14-18].

Due to the size of the device the use of LVAD for the treatment of end-stage HF was at first limited to patients with body surface areas >1.5 m², a condition only rarely fulfilled in women. In the analysis of the US National Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) among the 18 868 patients that underwent implantation of first continuous-flow LVAD, only 3984 (21.1 %) were women [19], despite a similar incidence of advanced HF in both sexes [1,2]. However, with the advance in LVAD technology (smaller size, the possibility of intrapericardial placement, etc.) it is now possible to use this treatment in patients with body surface of 1.2 m², which enables its application in women with end-stage HF.

The use of LVAD as a “bridge to recovery” is its most valuable application. Importantly, despite the presence of biological signs of LVAD-induced reverse remodeling [16], the functional myocardial recovery in patients with end-stage HF is very low: INTERMACS studies of unselected populations receiving LVAD showed that only 1-2 % of patients had their LVAD weaned due to satisfactory improvement of cardiac function. Approximately 12 % of patients showed

an increase in LV ejection fraction and normalization of LV dimension [16,20,21]. The reasons for this divergence between beneficial biological signs of reverse remodeling and lack of translation into clinical improvement remains unknown, but there is growing evidence that prolonged LVAD support can also exhibit detrimental effects [16,22]. Probably, the most harmful effect of long-term LVAD-induced mechanical unloading of the myocardium is cardiac atrophy. This may be the reason why the beneficial effects of mechanical unloading on biological features of the myocardium are not converted to functional improvement [23-28]. To minimize a deleterious outcome, various treatment approaches were evaluated, however, the attempts to minimize unloading-induced cardiac atrophy were only partly successful, which necessitates a further search for new therapies [24,29-32]. The prerequisite for some success is understanding the process of unloading-induced cardiac atrophy. To this purpose, the model of heterotopic rat HT_x onto the abdominal aorta of an isogenic rat recipient was developed and soon established in further relevant studies. Many scientific groups, including our own, have performed studies employing this model, and ample pertinent information has been obtained [26-28,32-41].

A major limitation of all such studies is that they were all performed in male animals only, hence the need to acquire information about the possible sex-related differences in the process of unloading-induced cardiac atrophy. This prompted us to elucidate if and what sex-related differences are present in the course of cardiac atrophy after heterotopic HT_x.

Methods

Ethical approval, animals, HT_x model, and gonadectomy technique

The studies were performed following guidelines and practices established by the Animal Care and Use Committee of the Institute for Clinical and Experimental Medicine, Prague, which accords with the European Convention on Animal Protection and Guidelines on Research Animal Use and were approved by this committee and subsequently by the Ministry of Health of the Czech Republic (the decision number for this project is 18680/2020-4/OVZ).

Adult male and female Lewis rats (Charles River Laboratories, Velaz, Prague, Czech Republic), 8 weeks of initial age, were used. The classical heterotopic HT_x, originally described by Ono and Lindsey [42] and

employed and validated by many investigators [27,28,33,35,37,38] was used as the model to simulate the effect of full mechanical unloading of the heart; its modification was established in our laboratory and is routinely employed [39-41].

Gonadectomy or sham-operation was performed under combined anesthesia of ketamine/midazolam mixture (Calypsol, Gedeon Richter, Hungary, 160 mg/kg of body weight and Dormicum, Roche, France 160 mg/kg of body weight, administered intraperitoneally), this was done 28 days before heterotopic HT_x. The details of the operation were as described in our previous studies [43,44]. Briefly, in female rats, the peritoneal cavity was opened and the ovaries and uterus were removed, thereafter the peritoneal cavity was cleaned and the muscle wall and the skin were sutured. In male rats, orchietomy was performed: the ductus deferens was isolated and ligated and then each testicle was removed *via* midline incision on the scrotum. Butorphanol (Torbugesic, Fort Dodge Animal Health, Fort Dodge, KS, USA), at the dose of 2 mg/kg of body weight, given every 12 h, was administered subcutaneously for 48-hour postoperative analgesia. In our earlier studies, the effectiveness of gonadectomy was validated within various time schedules (14, 28, and 56 days after gonadectomy) by determining plasma levels of testosterone and estradiol, assessed by radioimmunoassay [43,44].

Experimental design

The course of cardiac atrophy after heterotopic HT_x in healthy hearts: sex-differences and effects of castration

The experimental design used is outlined in Figure 1. Donor animals were anesthetized by inhalation of 2 % isoflurane (Forane, ABBVie Ltd., Prague, Czech Republic). Recipient animals were anesthetized with thiopental sodium (Thiopental, VUAB Pharma Ltd., Brno Czech Republic, 50 mg/kg of body weight intraperitoneally). We and others [27,35,37-41] have demonstrated that the unloading-induced cardiac atrophy develops within the first 14 days after HT_x when a dramatic loss of myocardial mass is seen. The following 40 days is a steady-state period, with no further loss of cardiac mass, suggesting stabilization of unloading-induced cardiac atrophy. Therefore, in the present study, the degree of cardiac atrophy was determined 7 days and 14 days after HT_x. The degree of atrophy was assessed from the weight of the total heart and of its individual structural components [LV + septum and right ventricle (RV)]. Explicitly, the index of cardiac atrophy was

calculated as the ratio of the weight of the heterotopically transplanted to the control heart (orthotropic native normal heart). The degree of cardiac atrophy was expressed as the percent decrease in the whole heart weight (HW), LV weight (LVW), and RV weight (RVW) of the hearts after HT_x compared to the control. Unfortunately, HW of the donor's heart before and after HT_x cannot be used for evaluation of the degree of cardiac atrophy, because the heart is immediately placed in cold cardioplegia solution, which precludes precise determination of HW. In order to obtain a better insight into the potential influence of sex hormones on the process of cardiac atrophy after HT_x, separate experimental groups were formed, and gonadectomy was performed in male and female recipient animals 28 days before HT_x; recipient animals without gonadectomy underwent sham-operation at the same time-point. The following groups were examined:

1. Sham-operated male Lewis rats (recipient) + HT_x of healthy donor's heart (7 days after HT_x) (n=9),
2. Sham-operated male Lewis rats + HT_x of healthy donor's heart (14 days) (n=10),
3. Castrated male Lewis rats + HT_x of healthy donor's heart (7 days) (n=9),
4. Castrated male Lewis rats + HT_x of healthy donor's heart (14 days) (n=9),
5. Sham-operated female Lewis rats + HT_x of healthy donor's heart (7 days) (n=9),
6. Sham-operated female Lewis rats + HT_x of healthy donor's heart (14 days) (n=9),
7. Castrated female Lewis rats + HT_x of healthy donor's heart (7 days) (n=10),
8. Castrated female Lewis rats + HT_x of healthy donor's heart (14 days) (n=9).

At the end of the experiment, the hearts were excised, blood was removed from the chambers by gentle compression, and the hearts' wet weight was determined.

In separate appropriately matched 8 experimental groups (n=10 in each), the hearts were subjected to histological examination of the myocardium as described previously [40,41,45,46].

Briefly, the rats were anesthetized with a combination of midazolam 5 mg.kg⁻¹ (Dormicum, Roche Ltd., Prague, Czech Republic) and ketamine 50 mg.kg⁻¹ (Calypsol, Gedeon Richter Ltd., Budapest, Hungary) *i.p.* Beating (pulsating) organs *i.e.* the native heart and the heart after HT_x (from the abdomen), were perfused *in situ* with 20 ml of Thomas cardioplegia solution and subsequently fixed in 4 % paraformaldehyde

in phosphate-buffered saline and embedded into Tis-sue-Tek. The blocks were cut using a cryomicrotome, and cardiomyocyte width was measured in the subendocardium, mid-myocardium, and sub-epicardium of the LV. Cardiomyocyte length was measured only in the mid-myocardium; in each layer, 50 cardiomyocytes were assessed. To avoid underestimation, only the cells in which the nucleus was visible were measured. Since there were no significant differences in the cardiomyocyte width between the layers, the data from the subendocardium, midmyocardium, and subepicardium were pooled as was also practiced by other investigators [47]. Analysis of LV and RV fibrosis was performed in sections stained with Picrosirius red (Direct Red 80, Sigma Aldrich, MO, USA) as described in detail previously [40,41,45]. Briefly, the interstitial collagen was analyzed in polarized light using 10 images of the LV and RV scanned from a mid-myocardium, without perivascular areas (magnification 200 \times , microscope Nikon eclipse Ni-E, camera Nikon DS-L3, Tokyo, Japan). The percent area of myocardial fibrosis was calculated semiquantitatively, using imaging software

NIS-Elements Ar (LIM, Prague, Czech Republic). The measurements of cardiomyocyte width and length, and of the degree of myocardial fibrosis were performed also 7 days and 14 days after heterotopic HT_x. The histological examination had to be performed in separate groups of animals because perfusion with cardioplegia solution with subsequent immediate fixation in paraformaldehyde solution precludes precise determination of the whole HW and the LV or RV weights.

Statistical analyses

All values are expressed as mean \pm SEM. Using the Graph-Pad Prism software (Graph Pad Software, San Diego, CA, USA), statistical analysis was done by Wilcoxon's signed-rank test for unpaired data, or one-way analysis of variance (ANOVA) when appropriate. ANOVA analysis was employed for evaluation of the differences within the same experimental group over time (i.e. changes on days 7 and 14 after HT_x). Values exceeding the 95 % probability limits ($p < 0.05$) were considered statistically significant.

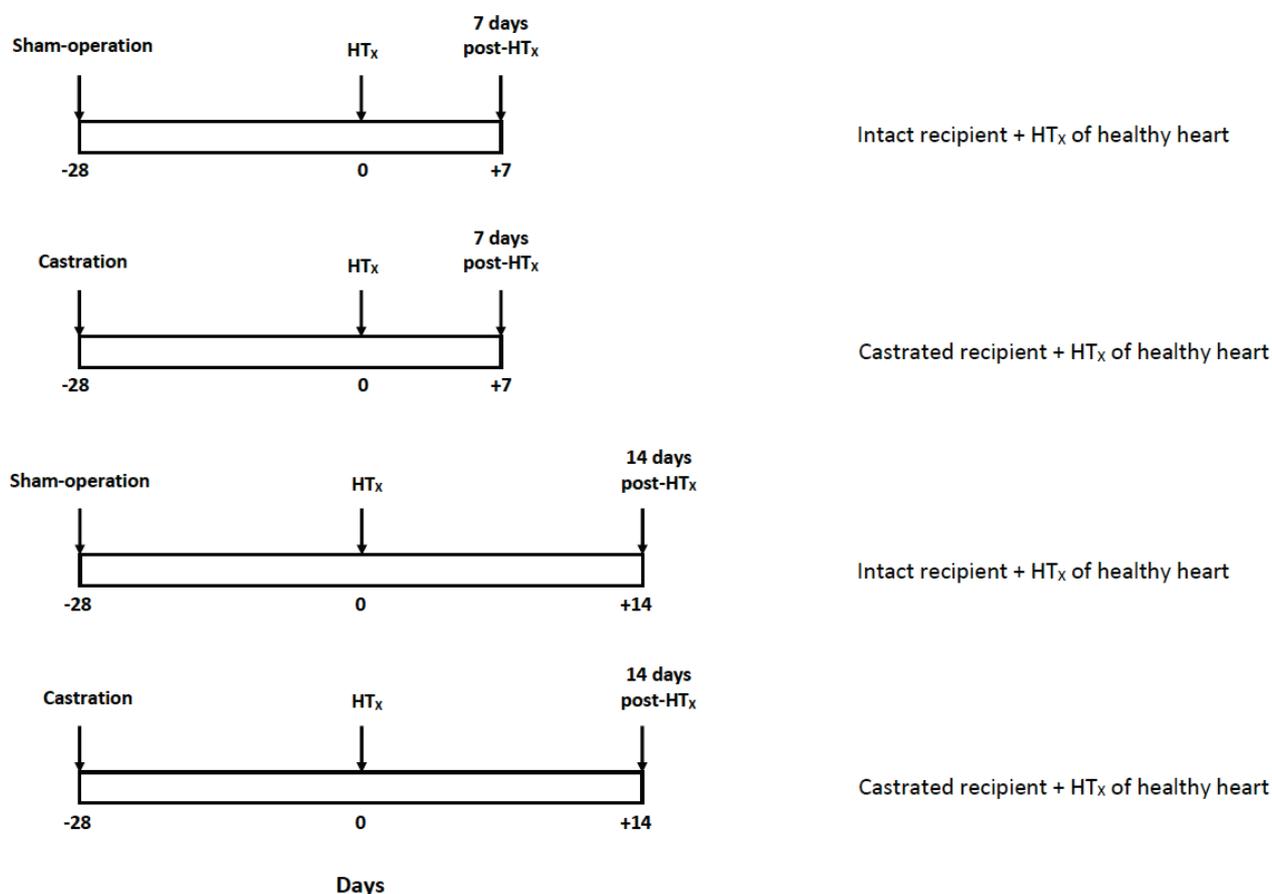


Fig. 1. An outline of the set-up of experimental groups in male and female Lewis rats. HT_x denotes heterotopic heart transplantation.

Results

Table 1 summarizes the values of body weight (BW), tibial length (TL), HW, LVW, and RVW of native (i.e. orthotopic, in the chest of the recipient) and transplanted (i.e. heterotopic) hearts in absolute values, measured 7 days and 14 days after HT_x. Gonadectomy in female rats resulted in a significant rise in BW when compared to intact females at the same time point, however, no significant change was seen in male rats. Furthermore, gonadectomy itself did not alter TL, suggesting that it did not modify the general growth of the rat, and it did not change the weight of the native hearts or its individual structural components, similarly in male and female rats at any time point, suggesting that it also did not alter the heart growth.

As shown in Figure 2A, 7 days after mechanical unloading by HT_x, intact male rats showed a significantly greater decrease in whole HW compared to intact female rats (-28±1 vs. -13±1 %, $p<0.05$), the decrease further progressed till day 14 in intact male rats (-45±1 %), but there was no further progress in intact female rats (-12±1 %). The dynamics of LV and RV atrophy were quite similar as in whole hearts (Fig. 2B, C). The data of Figure 2 show that at no time point did gonadectomy have any significant effect on the decreases in whole HW, LVW, and RVW, similarly in male and female rats.

Figure 3A shows the whole HW values in the native hearts normalized to tibial length (TL). This is the standard way to assess cardiac mass in groups of animals with significant differences in BW [48,49,50]. The values of HW/TL ratio show that male rats (both intact and after gonadectomy), at all-time points, have higher cardiac mass of native hearts when compared to those of female rats; this was also the case with the difference between the rats that were intact or underwent gonadectomy. As shown in Figure 3B, 7 days after HT_x the augmented decreases in whole HW after mechanical unloading by HT_x resulted in a similar ratio of HW to TL in male and female rats, and 14 days after HT_x this ratio was even significantly higher in the female rats when compared to the male rats. Gonadectomy did not modify this ratio in any of the experimental groups at any time point.

Similar results were obtained when the ratio of LVW to TL was analyzed (data not shown).

Table 2 summarizes the parameters of myocyte size, specifically cardiomyocyte length (CL), cardiomyocyte width (CW), and the ratio of CL to CW of native and transplanted hearts in absolute values,

measured 7 and 14 days after HT_x. As shown, there were no significant differences between males and females in the CL, CW, and the CL to CW ratio in the native hearts, and gonadectomy did not alter these parameters, similarly in male and female rats.

As shown in Figure 4A, mechanical unloading by HT_x after 7 days and 14 days resulted in similar decreases in CL in intact male and female rats, and gonadectomy did not have any significant effect (at all-time points) on the decreases in CL, both in male and female rats. In contrast, 14 days after HT_x mechanical unloading by HT_x resulted in significantly greater decreases in CW in intact males when compared to intact females (-35±3 vs. -14±3 %, $p<0.05$). There was a tendency for a greater decrease in CW already 7 days after HT_x, but the change was not significant. Gonadectomy did not modify the course of changes in CM, similarly in male and female rats (Fig. 4B).

As shown in Figure 4C, the augmented decreases in CW in intact male rats led to an increase in the CL to CW ratio in intact male rats compared to intact female rats 14 days after HT_x (+26±5 vs. -9±3 %, $p<0.05$). Gonadectomy did not alter the CL to CW ratio, similarly in male and female rats.

Figure 5 summarizes the data on the index of myocardial fibrosis (expressed in %) in the LV (Fig. 5A) and RV (Fig. 5B). There were no significant differences in the myocardial fibrosis in the LV or the RV in the native hearts, similarly in male and female rats (at all-time points). Gonadectomy did not alter the degree of myocardial fibrosis in either ventricle of native hearts, similarly in male and female rats.

As shown in Figure 5A, 7 days after mechanical unloading by HT_x the degree of fibrosis in the LV was significantly higher in transplanted hearts of intact male rats and intact female rats when compared to native hearts, and it markedly progressed when assessed 14 days after HT_x. However, the degree of myocardial fibrosis in the LV was significantly lower in the transplanted hearts of intact female rats when compared to intact male rats, when assessed 14 days after HT_x. Gonadectomy did not modify the development of myocardial fibrosis in the LV of male and female rats.

The development of myocardial fibrosis in the RV after mechanical unloading by HT_x showed a similar pattern as observed in the LV (Fig. 5B). However, the degree of myocardial fibrosis in the RV in intact male rats was significantly higher than observed in the LV (27.28±1.83 vs. 17.78±1.65 %, $p<0.05$). In addition, in

contrast to the situation seen in the LV, gonadectomy significantly reduced the degree of myocardial fibrosis in the RV of male rats, when compared to their intact counterparts (18.04 ± 1.21 vs. 27.28 ± 1.83 % $p < 0.05$) (Fig. 5B). Gonadectomy did not alter the course of myocardial fibrosis in the RV of transplanted hearts in

female rats (Fig. 5B).

Representative images of myocardial fibrosis in the LV of the native (i.e. orthotropic) and transplanted (i.e. heterotopic) hearts are shown in Figure 6, and those for the RV are shown in Figure 7.

Table 1. The weight of the native heart (i.e. recipient heart) and the transplanted heart (i.e. donor's heart) and of the individual structural components after heterotopic heart transplantation (HT_x). Native heart values served as basal values (100 %) for evaluation of the process of cardiac atrophy in animals after HT_x .

	BW (g)	TL (mm)	Parameter					
			HW (mg) (native)	HW (mg) (HT_x)	LVW (mg) (native)	LVW (mg) (HT_x)	RVW (mg) (native)	RVW (mg) (HT_x)
Groups of males								
<i>Intact male recipient + HT_x of male donor's heart (7 days after HT_x)</i>	365±11	40.0±0.3	1013±25	725±19	657±24	456±12	173±13	127±8
<i>Intact male recipient + HT_x of male donor's heart (14 days after HT_x)</i>	384±7	40.8±0.1	1033±21	571±19	686±16	369±16	179±8	104±6
<i>Castrated male recipient + HT_x of male donor's heart (7 days after HT_x)</i>	376±7	40.5±0.2	1022±21	683±19	690±22	443±18	181±6	135±5
<i>Castrated male recipient + HT_x of male donor's heart (14 days after HT_x)</i>	384±4	40.6±0.2	951±14	548±12	646±12	368±12	177±5	102±4
Groups of females								
<i>Intact female recipient + HT_x of female donor's heart (7 days after HT_x)</i>	216±2*	35.1±0.1*	746±11*	647±11	537±3*	445±5	137±5*	121±4
<i>Intact female recipient + HT_x of female donor's heart (14 days after HT_x)</i>	228±2*	35.6±0.2*	766±19*	668±7*	484±15*	432±9*	120±8*	106±7
<i>Castrated female recipient + HT_x of female donor's heart (7 days after HT_x)</i>	261±3*.#	36.3±0.2*	786±21*	674±11	519±11*	449±11	156±8*	133±5
<i>Castrated female recipient + HT_x of female donor's heart (14 days after HT_x)</i>	267±3*.#	36.6±0.1*	759±16*	653±10*	508±9*	442±3*	135±9*	121±8

Values are means ± SEM. BW, body weight; TL, tibia length; HT_x , heterotopic heart transplantation; HW, heart weight; LVW, left ventricle weight; RVW, right ventricle weight. * $P < 0.05$ vs. male at the same day after HT_x (i.e. effects sex differences on the followed parameter). # $P < 0.05$ vs. castrated male at the same day after HT_x (i.e. effects of castration on the followed parameter)

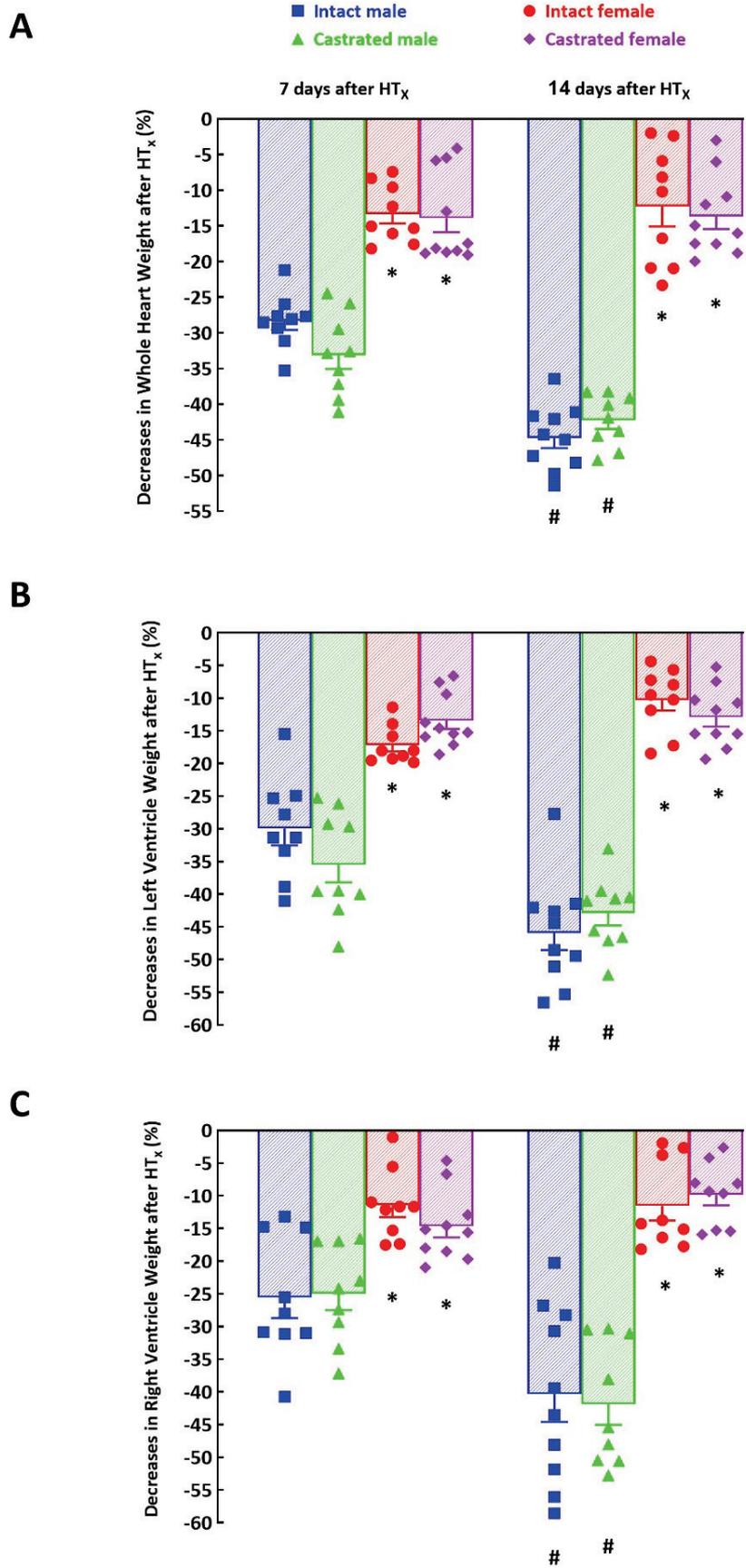


Fig. 2. Effects of castration on the course of cardiac atrophy in response to mechanical heart unloading induced by heterotopic heart transplantation (HT_x) in male and female Lewis rats. Data are expressed as percent decreases compared with the native heart: **(A)** changes in whole heart weight, **(B)** changes in left ventricle weight, **(C)** changes in right ventricle weight. * P<0.05 compared with male animals at the same time point. # P<0.05 compared with the values of animals studied 7 days after HT_x.

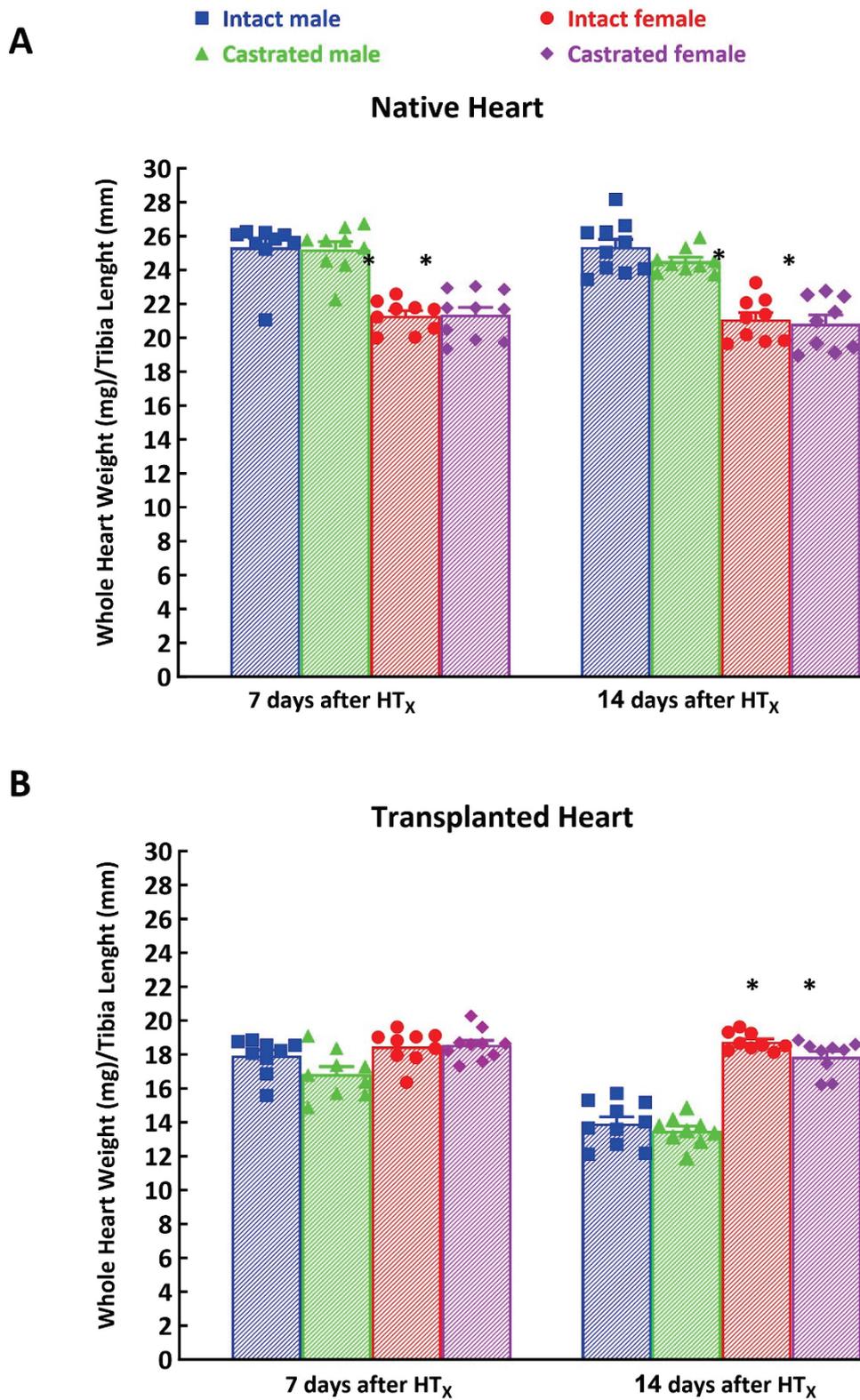


Fig. 3. Effects of castration on the course of whole heart weight to tibia length ratio 7 days and 14 days after heterotopic heart transplantation (HT_x) in male and female Lewis rats: **(A)** values in native (i.e. orthotopic) heart, **(B)** values in transplanted (i.e. heterotopic) heart. * $P < 0.05$ compared with male animals at the same time point.

Table 2. Cardiomyocyte length and cardiomyocyte width of the native heart (i.e. recipient heart) and the transplanted heart (i.e. donor's heart) of the left ventricle after heterotopic heart transplantation (HT_x). Native heart values served as baseline (100 %) for evaluation of the process of cardiac atrophy in animals after HT_x.

	Parameter					
	CL (μm) (native)	CL (μm) (HT _x)	CW (μm) (native)	CW (μm) (HT _x)	CL/CW (native)	CL/CW (HT _x)
Groups of males						
<i>Intact male recipient + HT_x of male donor's heart (7 days after HT_x)</i>	127.1±1.71	105.1±2.33	15.76±0.31	13.73±0.73	8.09±0.17	7.83±0.45
<i>Intact male recipient + HT_x of male donor's heart (14 days after HT_x)</i>	123.8±0.89	99.5±2.21	16.28±0.44	10.56±0.58	7.65±0.19	9.46±0.48
<i>Castrated male recipient + HT_x of male donor's heart (7 days after HT_x)</i>	122.1±1.2	111.2±2.46	15.58±0.25	13.34±0.33	7.85±0.13	8.37±0.36
<i>Castrated male recipient + HT_x of male donor's heart (14 days after HT_x)</i>	120.7±1.8	102.8±2.62	16.77±0.34	11.94±0.27	7.21±0.11	8.66±0.35
Groups of females						
<i>Intact female recipient + HT_x of female donor's heart (7 days after HT_x)</i>	114.7±1.62	101.1±1.41	15.29±0.75	14.47±0.53	7.61±0.31	7.06±0.30
<i>Intact female recipient + HT_x of female donor's heart (14 days after HT_x)</i>	118.2±1.92	89.1±1.25	16.09±0.24	11.94±0.27	7.35±0.14	6.67±0.28*
<i>Castrated female recipient + HT_x of female donor's heart (7 days after HT_x)</i>	114.4±1.83	97.2±1.71	16.36±0.47	15.12±0.42*	7.04±0.21	6.45±0.13*
<i>Castrated female recipient + HT_x of female donor's heart (14 days after HT_x)</i>	115.1±1.63	92.2±2.32	16.38±0.47	13.91±0.39*	7.09±0.27	6.65±0.17*

Values are means ± SEM. CL, cardiomyocyte length; CW, cardiomyocyte width; HT_x, heterotopic heart transplantation. *P<0.05 vs. male at the same day after HT_x (i.e. effects sex differences on the parameter measured).

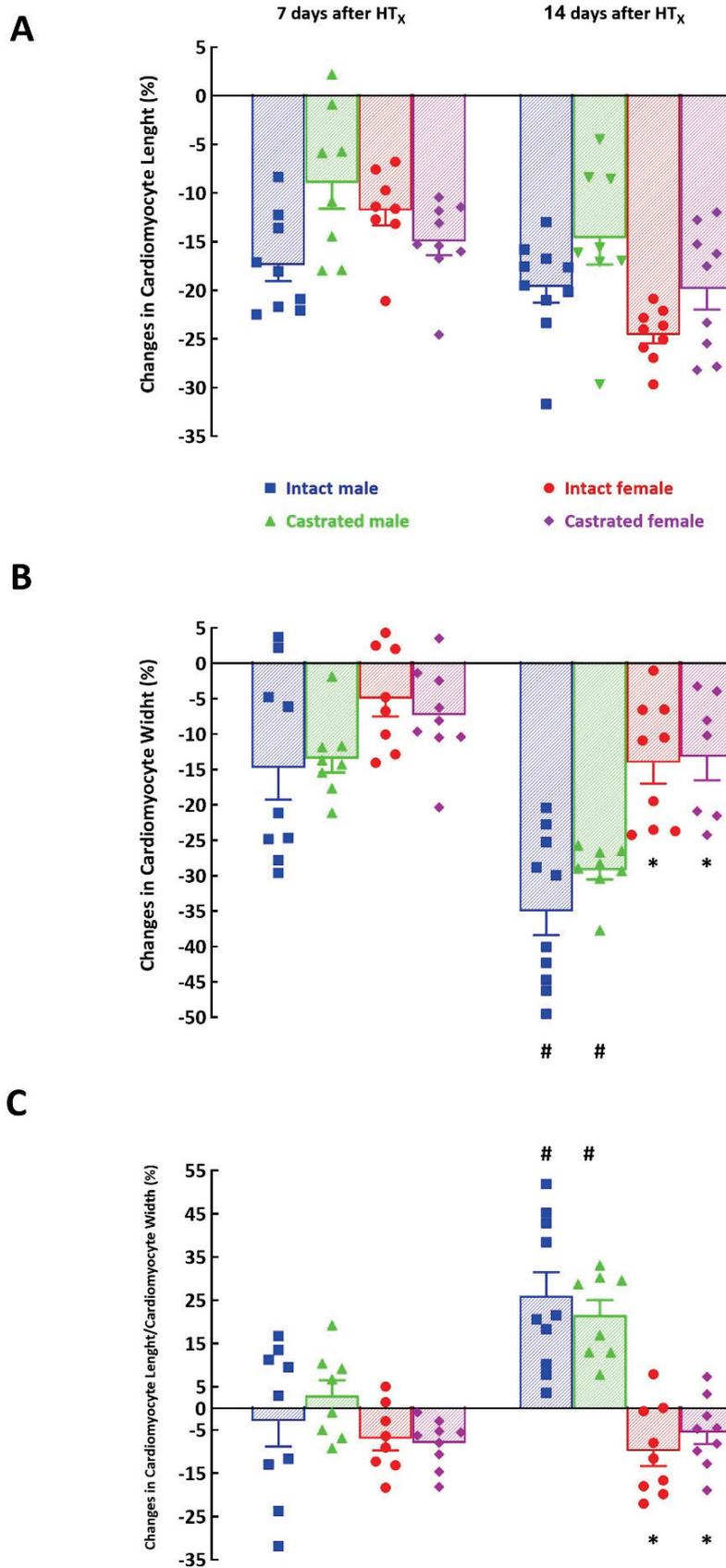


Fig. 4. Effects of castration on the course of myocyte size in the left ventricle in response to mechanical heart unloading induced by heterotopic heart transplantation (HT_x) in male as well as in female Lewis rats. Data are expressed as percent decreases compared with the native heart: **(A)** changes in cardiomyocyte length, **(B)** changes in cardiomyocyte width, **(C)** changes in cardiomyocyte length to width ratio. *P<0.05 compared with male animals at the same time point. #P<0.05 compared with the values of animals studied 7 days after HT_x.

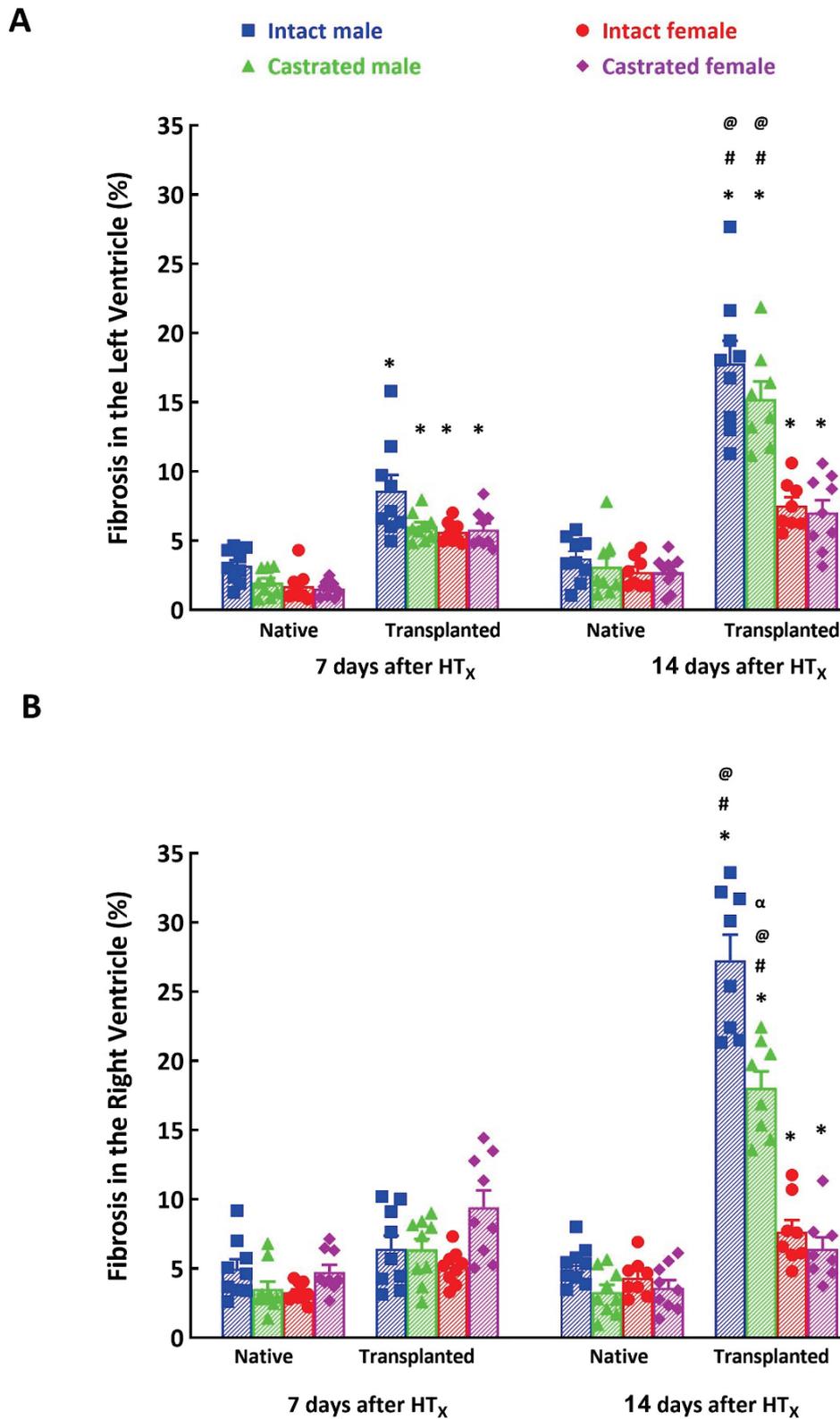


Fig. 5. Effects of castration on the index of myocardial fibrosis for the left ventricle (**A**) and the right ventricle (**B**) of the native heart, and in response to mechanical heart unloading induced by heterotopic heart transplantation (HT_x) in males as well as in female Lewis rats 7 and 14 days after HT_x. * P<0.05 compared with values from native hearts at the same time point and the same experimental group. # P<0.05 compared with values from transplanted hearts studied 7 days after HT_x. @ P<0.05 compared with values from transplanted hearts studied 14 days after HT_x in female Lewis rats. ° P<0.05 compared with the values from transplanted hearts studied 14 days after HT_x in intact male Lewis rats.

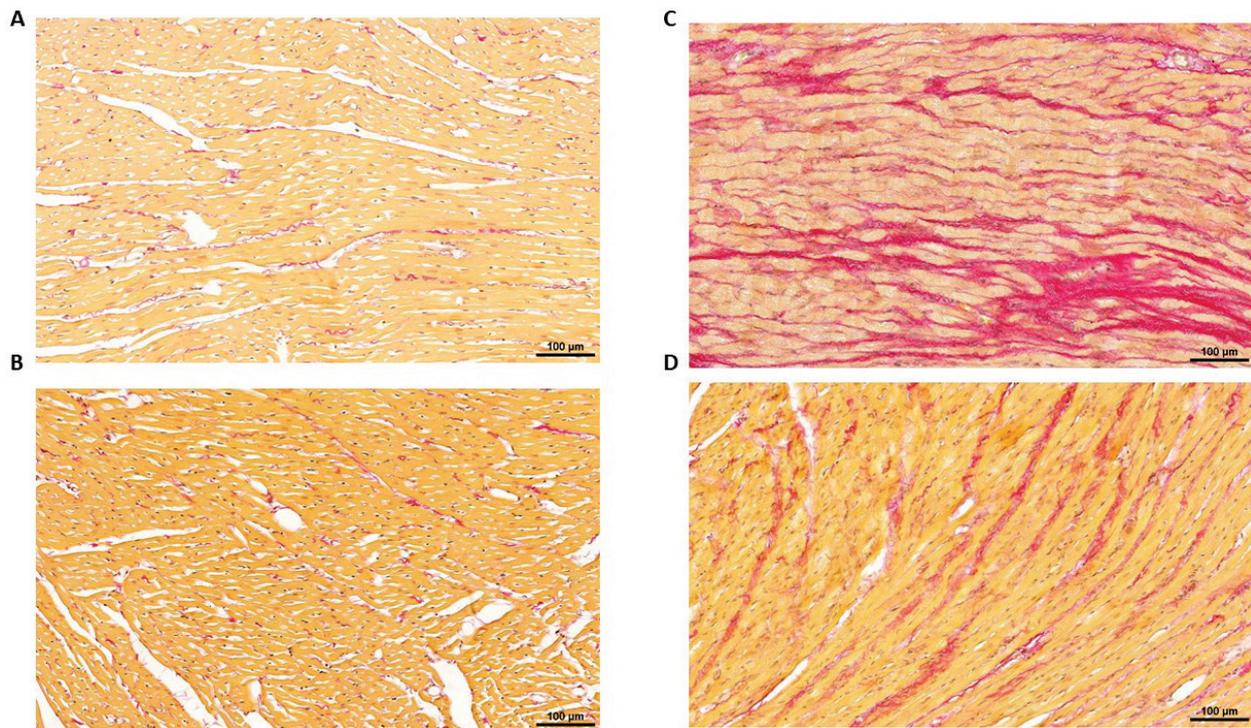


Fig. 6. Representative histological images of the left ventricle from the native heart (i.e. recipient's heart) and from the transplanted heart 14 days after heterotopic heart transplantation. Sections are stained with Picrosirius Red (200×), in these bright-field microscopy images the collagen is red against a pale yellow background. The scale bar in the figure is 100 µm. **(A)** Intact native heart from male Lewis rats (average fibrosis in this group is 3.69 ± 0.53 %), **(B)** Intact native heart from female Lewis rats (average fibrosis in this group is 2.71 ± 0.39 %), **(C)** Intact transplanted heart from male Lewis rats (average fibrosis in this group is 17.78 ± 1.65 %), **(D)** Intact transplanted heart from female Lewis rats (average fibrosis in this group is 7.52 ± 0.62 %).

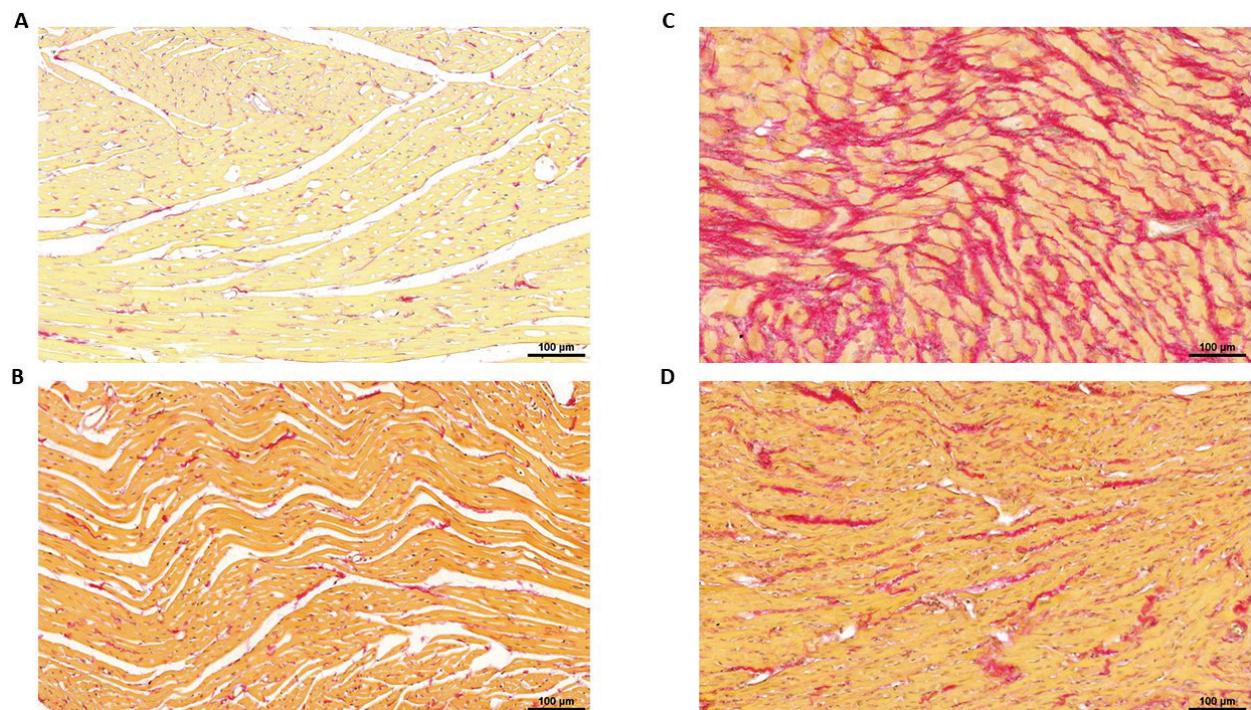


Fig. 7. Representative histological images of the right ventricle from the native heart (i.e. recipient's heart) and from the transplanted heart 14 days after heterotopic heart transplantation. Sections are stained with Picrosirius Red (200×), in these bright-field microscopy images the collagen is red against a pale yellow background. The scale bar in the figure is 100 µm. **(A)** Intact native heart from male Lewis rats (average fibrosis in this group is 5.24 ± 0.46 %), **(B)** Intact native heart from female Lewis rats (average fibrosis in this group is 4.71 ± 0.48 %), **(C)** Intact transplanted heart from male Lewis rats (average fibrosis in this group is 27.28 ± 1.83 %), **(D)** Intact transplanted heart from female Lewis rats (average fibrosis in this group is 7.66 ± 0.85 %).

Discussion

The first important set of findings of the present study relates to our observation that the development of unloading-induced cardiac atrophy is substantially less significant in female than in male rats. We observed smaller decreases in whole HW, LVW, and RVW of the transplanted heart in the females (Fig. 2). Greater decreases in whole HW after HT_x are reflected by a higher HW to TL ratio of the transplanted heart in the female than in male rats. This index is initially higher in male rats, as apparent from data obtained from native hearts (Table 1 and Fig. 3). All this suggests that attenuation of unloading-induced cardiac atrophy in female rats results in greater cardiac mass (when normalized to TL) in the transplanted heart of female rats than observed in male rats at the end of the study. In addition, we saw that mechanical unloading induced by HT_x also reduced the decrease in CW in female rats. This data suggests that attenuation of unloading-induced cardiac atrophy in female rats was apparent also at the cardiomyocyte level.

In this context, in accordance with the basimetric approach, i.e. with the law of initial value in biological sciences [51] which states that the relative (i.e. percentage) change from the initial level largely depends on the latter value, one explanation for the greater unloading-induced cardiac atrophy in male rats might be higher initial cardiac mass. However, the finding of similar CW in male and female rats in the native heart, which represents an initial (basal) level for the evaluation of cardiac atrophy in animals after HT_x (Table 2), and particularly our finding that mechanical unloading by HT_x resulted in significantly greater decreases in CW in male rats, argues against the notion that augmented unloading-induced cardiac atrophy in male rats is a simple consequence of higher basal values of cardiac mass.

Furthermore, our findings show that HT_x-induced mechanical unloading resulted in the development of marked myocardial fibrosis in the LV as well as RV, but the fibrosis was substantially greater in male rats (2× greater in LV and 3× greater in RV, Fig. 4). Our present findings are in accordance with previous studies (performed exclusively in male animals) showing a gradual increase of myocardial fibrosis in the LV and RV after HT_x-induced mechanical unloading. This increase was believed to be a consequence of activation of a fibrotic remodeling program and increased

concentration of collagen and it was claimed that subsequent myocardial process is an exclusively maladaptive process [28,32,33,45,52,53]. However, the view that myocardial fibrosis is a solely detrimental maladaptive process has been challenged because besides the “interstitial fibrosis” and “perivascular fibrosis” that undoubtedly represent maladaptive processes, there exist also “replacement fibrosis” that reflects a reparative process [54]. The “replacement fibrosis” in myocardial infarction is a typical example, because sudden death of a large number of cardiomyocytes is followed by such reparative response and helps preserve structural integrity of the chamber after myocardial injury; this may prevent catastrophic mechanical complications, such as cardiac rupture [54]. Therefore, the observed increase in myocardial fibrosis in the LV and RV after HT_x-induced mechanical unloading might be an alternative straightforward explanation why the loss of cardiomyocyte mass leads to increased “passive myocardial fibrosis”. Such response would depend on a much longer half-life of collagen degradation, which resembles the “replacement fibrosis”. This notion is supported by findings that despite increased myocardial fibrosis in transplanted hearts the stiffness is not increased when compared with native hearts [33-38,45,52,53].

The second important set of findings relates to the potential role of sex hormones on the differences in the process of unloading-induced cardiac atrophy. We found that gonadectomy did not alter the course of HT_x-induced cardiac atrophy in male or female rats. Moreover, gonadectomy did not modify myocardial fibrosis in the LV as well as the RV of the transplanted hearts, both in female rats and in the LV of the male rats. On the contrary, castration of male rats attenuated myocardial fibrosis when measured 14 days after HT_x. Therefore we conclude that the substantial sex-related differences in the course of cardiac atrophy and myocardial fibrosis after mechanical unloading cannot be simply ascribed to the deleterious actions of testosterone or to the protective effects of estradiol. We are aware that this conclusion is valid only for the so-called “activational effects of sex hormones”, which disappears with the removal of sex hormones, and not for “organizational effects of sex hormones”, which persist in the absence of sex hormones because they are based mainly on epigenetic modification of DNA [11]. Of interest are the beneficial effects of castration on the degree of myocardial fibrosis in the RV of male rats; this indicates some ventricle-related differences in the

response to sex hormones in this process after HT_x. This notion is additionally supported by our findings that in male rats the degree of myocardial fibrosis in the RV is markedly higher than in the LV whereas in female rats it is almost identical in both ventricles. Admittedly, our present experimental design does not enable any deeper assessment of those ventricle-related HT_x-induced differences in the development of myocardial fibrosis in male rats and future studies are needed with particular focus on the role of sex hormones in the development of myocardial fibrosis in the RV of male rats.

Our conclusion that sex-linked differences in the course of unloading-induced cardiac atrophy cannot be simply ascribed to the presence of sex hormones is based on the classical experimental approach to explore sex-linked differences, i.e. on the comparison of intact and gonadectomized animals [11,54]. To arrive at a definite conclusion, comprehensive studies would be needed in order to evaluate the process of cardiac atrophy and myocardial fibrosis after mechanical unloading induced by HT_x in animals after gonadectomy, in those after gonadectomy with substitution of appropriate hormones, as well as those after gonadectomy with the administration of steroid hormones of the opposite sex [11,55]. Since it is not clear if the sex-linked differences in the course of unloading-induced cardiac atrophy, as seen in the present study, are due to the inherent properties of the donor's heart or the hormonal homeostatic conditions of the recipient, an alternative approach would be to investigate the response of the female heart transplanted into a male recipient and vice versa. Of course, all these animal groups should be subjected to experimental protocols identical to those in the present study. Understandably, the aforementioned required studies are extremely demanding and exceptionally

difficult to interpret. Nevertheless, we are deeply convinced that our present results provide sufficient evidence for the conclusion that the development of unloading-induced cardiac atrophy is substantially attenuated in female rats when compared to male rats.

Perspectives and significance

Our present data clearly show that the development of unloading-induced cardiac atrophy and myocardial fibrosis is substantially attenuated in females as compared with male rats, and these differences cannot be simply ascribed to the presence of sex steroid hormones. Based on the foregoing discussion, we conclude that cardiac atrophy, the main detrimental effect of mechanical unloading by HT_x is reduced in females as compared with male rats. This information derived from our present experimental study should be considered in attempts to develop new therapeutic measures aimed at enhancing myocardial recovery in patients on prolonged LVAD support.

Conflict of Interest

There is no conflict of interest.

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