Sex-Linked Differences in Cardiac Atrophy After Heterotopic Heart Transplantation: No Direct Relation to the Actions of Sex Steroid Hormones

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Summary

An important complication of prolonged support of the left ventricle with an assist device when implanted in patients with heart failure is unloading-induced cardiac atrophy. Our recent study suggested that sex-linked differences in the development of atrophy induced by heterotopic heart transplantation (HT_X) do exist, however, the role of the environmental conditions dependent on plasma concentrations of sex hormones remains elusive. We aimed to compare the course of HT_X-induced cardiac atrophy in male and female rats after gonadectomy with substitution of steroid hormones of the opposite sex. In a separate series of experiments, we evaluated the course of unloading-induced cardiac atrophy in the female heart transplanted into a male recipient and vice versa. Cardiac atrophy was assessed as the ratio of the transplanted heart weight to native heart weight (HW), which was determined 14 days after HT_X. In female rats, studied in both experimental variants, HT_x resulted in significantly smaller decreases in whole HW when compared to those observed in male rats exposed to the same experimental conditions (-9 ± 1 and - 11 + 1 vs. -44 ± 2 and -42 ± 2 %, $p<0.05$ in both cases). The dynamic of changes in left and right ventricle was similar as in the whole HW. Our results show that the process of unloading-induced cardiac atrophy exhibits important sex-linked differences and that attenuation of this process in female rats cannot be simply ascribed to the protective effects of estradiol or to the absence of deleterious actions of testosterone.

Keywords

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

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Introduction

Heart transplantation (HT_x) is the best therapeutic approach for the treatment of patients with end-stage heart failure (HF), however, the scarcity of organ donors limits the number of HT_x performed. Therefore, implantation of the left ventricle assist devices (LVADs) has emerged as an alternative treatment for patients with end-stage HF [1-4]. However, the most harmful effect of long-term LVAD use is probably the development of cardiac atrophy, a consequence of LVAD-induced mechanical unloading. It has been claimed that this may be one of the reasons why the beneficial effects on the biological features of the myocardium have not been so far translated into functional improvement [5-15]. Attempts to minimize unloading-induced cardiac atrophy were usually

unsuccessful, which further necessitates a search for new treatment strategies [8,12,13,16-20]. The prerequisite for finding a treatment approach that would minimize unloading-induced cardiac atrophy is profound understanding the physiology of the process. To meet this need, a model of heterotopic rat HT_x onto the abdominal aorta of an isogenic rat recipient was developed. Many research groups, including our own, performed studies employing this model, which provided ample relevant information [5,9,20-31]. The critically important limitation of such studies is the fact that they were performed in male animals only. Since it is known that there are important sex-related differences in the pathophysiology of HF [32-35], one should also expect the presence of such differences in the process of unloading-induced cardiac atrophy. This prompted us recently to elucidate if, and to what extent, sex-related differences are present in the course of cardiac atrophy after heterotopic HT_x. We found that the development of unloading-induced cardiac atrophy was substantially less pronounced in female than in male rats, and that gonadectomy did not alter the course of HT_x-induced cardiac atrophy, similarly in male and female rats. We concluded that the development of unloading-induced cardiac atrophy is less pronounced in female than in male rats, and that those sex-linked differences were not caused by the activity of sex hormones [36]. However, an ultimate conclusion should not be exclusively based on the classical experimental approach (comparison of intact animals with those after gonadectomy) but also on evaluation of the course of HT_x-induced cardiac atrophy in subjects after gonadectomy with substitution of sex steroid hormones of the opposite sex [34,37,38]. Evidently, an alternative approach should be sought to answer the question if the sex-linked differences in the course of unloading-induced cardiac atrophy are due to the inherent properties of donor´s (i.e. transplanted) heart or to the hormonal environment of the recipient. Therefore one should investigate the response of the female heart transplanted into a male recipient and vice versa. Accordingly, the aim of the present study was to assess sex-linked differences in the development of unloading-induced cardiac atrophy using both aforementioned approaches.

Methods

Ethical approval

The studies were performed in agreement with the guidelines and practices established by the *Animal Care and Use Committee of the Institute for Clinical and Experimental Medicine*, Prague, which accord 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).

Animals and HT_x model

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 [39] and employed and validated by many investigators [9,21,23,25,27,28] 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 [29-31,36].

Gonadectomy technique

Gonadectomy or sham-operation was performed under combined anesthesia with intraperitoneal ketamine/midazolam mixture (Calypsol, Gedeon Richter, Hungary, 160 mg/kg of body weight, and Dormicum, Roche, France 160 mg/kg of body weight), this was done 28 days before heterotopic HT_x. The details of the operation were as described in our previous studies [40,41]. 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 skin were sutured. In male rats, orchiectomy 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 hours, was administered subcutaneously for 48-hour postoperative analgesia. In our earlier studies, the effectiveness of gonadectomy was validated by determining plasma levels of testosterone and estradiol, assessed by radioimmunoassay [40,41].

Hormonal substitution

The experimental design for evaluation of the effectiveness of hormonal substitution with steroid hormones of the opposite sex is outlined in Fig. 1. It shows that female rats were gonadectomized on the day labeled -28 and the substitution with testosterone was immediately initiated and repeated on the day labeled 0; the experiment ended on the day labeled +14. Plasma

levels of testosterone were assessed throughout the study as outlined in Fig. 1A and compared with sham-operated males that served as controls. Fig. 1B shows the experimental design for evaluation of the effectiveness of hormonal substitution by estradiol in goadectomized male rats, which was identical as the design used for testosterone substitution in gonadectomized female rats.

Due to the fact that repeated determination of plasma testosterone and estradiol requires a large volume of blood (400 µl), these assessments were performed initially in separate groups of animals (n = 8 in each). Plasma levels of estradiol and testosterone were assessed by RIA techniques as described in our previous studies [40,41]. The following experimental groups were examined:

1) Sham-operated (i.e., Intact) male Lewis rats
2) Sham-operated (i.e., Intact) female Lewis rats
3) Castrated male Lewis rats
4) Castrated female Lewis rats
5) Castrated female Lewis rats + Testosterone substitution
6) Castrated male Lewis rats + Estradiol substitution

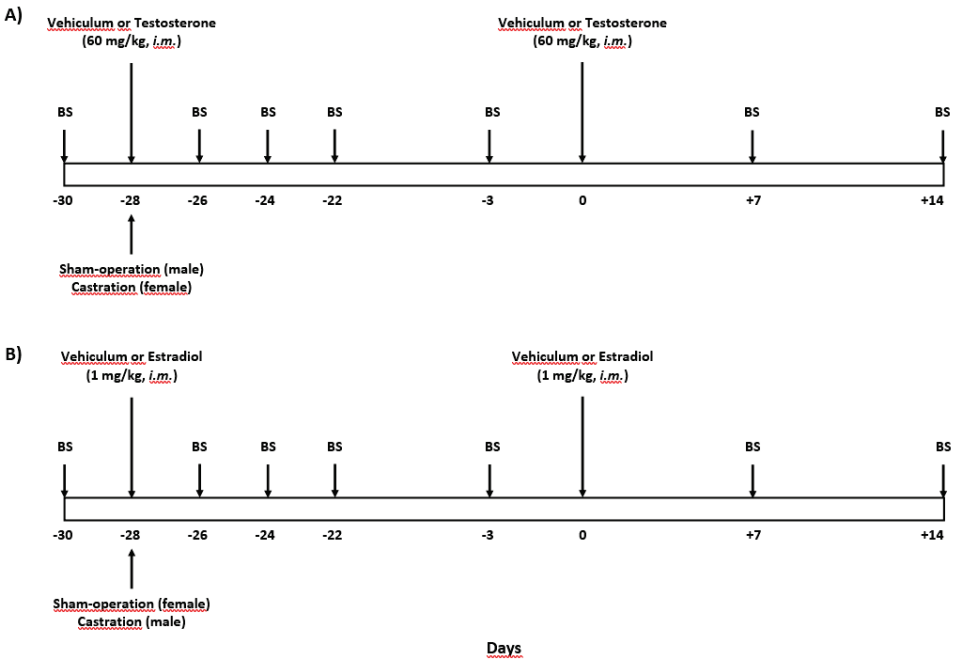

Fig. 1. An outline of the set of experiments in male and female Lewis rats performed for evaluation of the effectiveness of hormonal substitution with steroid hormones of the opposite sex. BS indicates blood sampling from the tail vein.

Detailed experimental design for evaluation of unloading-induced cardiac atrophy.

Series 1: The course of cardiac atrophy after heterotopic HTx in rats after castration and substitution of steroid hormones of the opposite sex

The experimental design used is outlined in Fig. 2. Donor animals were anesthetized by inhalation of 2 % isoflurane (Forane, ABBVie Ltd., Prague, Czech Republic) and the hearts were harvested and transplanted as described previously [29-31,36]. Recipient animals were anesthetized with intraperitoneal thiopental sodium (Thiopental, VUAB Pharma Ltd., Brno Czech Republic, 50 mg/kg of body weight). We and others [9,25,27-31] 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 14 days after HT_x. Recipient animals were castrated and the supplementation of steroid sex hormones of the opposite sex was started and performed as described above and validated in the initial above-described studies. The degree of atrophy was assessed from the total heart weight and of its individual structural components [i.e. left ventricle (LV) + septum and right ventricle (RV)]. Explicitly, the index of cardiac atrophy was calculated as the ratio of the weight of the heterotopically transplanted heart to the recipient native normal heart. The degree of cardiac atrophy was expressed as percent decrease in the whole heart weight (HW), LV weight (LVW), and RV weight (RVW) of the hearts after HT_x. 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 donor´s heart is immediately placed in cold cardioplegia solution, which precludes precise determination of HW. The following experimental groups were examined:

1. Castrated male Lewis rats (recipient) + estradiol substitution + HT_x of healthy male donor´s heart (14 days after HT_x) (n = 10),
2. Castrated female Lewis rats (recipient) + testosterone substitution + HT_x of healthy female donor´s heart (14 days after HT_x) (n = 10),

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.

Series 2: The course of cardiac atrophy after heterotopic HTx to the recipient of the sex opposite to that of the donor´s heart

The experimental design is outlined in Fig. 3 and was virtually the same as described for series 1. However, native heart of the recipient cannot be used as the control for calculation of the index of cardiac atrophy. Evidently, the hearts of male's recipient cannot serve as controls (i.e. 100 %) for the hearts of female´s donor´s heart and vice versa, due to the differences in cardiac mass between males and females. Therefore, the hearts from males and females without HT_x were used as controls (Figs 3C, D). The following experimental groups were examined:

1. Intact female Lewis rats (recipient) + HT_x of healthy male donor´s heart (14 days after HT_x) (n = 10),
2. Intact male Lewis rats (recipient) + HT_x of healthy female donor´s heart (14 days after HT_x) (n = 10),
3. Intact male Lewis rats without HT_x (14 days after sham-operation) (n = 10),
4. Intact female Lewis rats without HT_x (14 days after sham-operation) (n = 10).

Statistical analyses

All values are expressed as mean ± 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 differences in plasma concentrations of steroid hormones within the same experimental group over time. The values exceeding 95 % probability limits ($p<0.05$) were considered statistically significant.

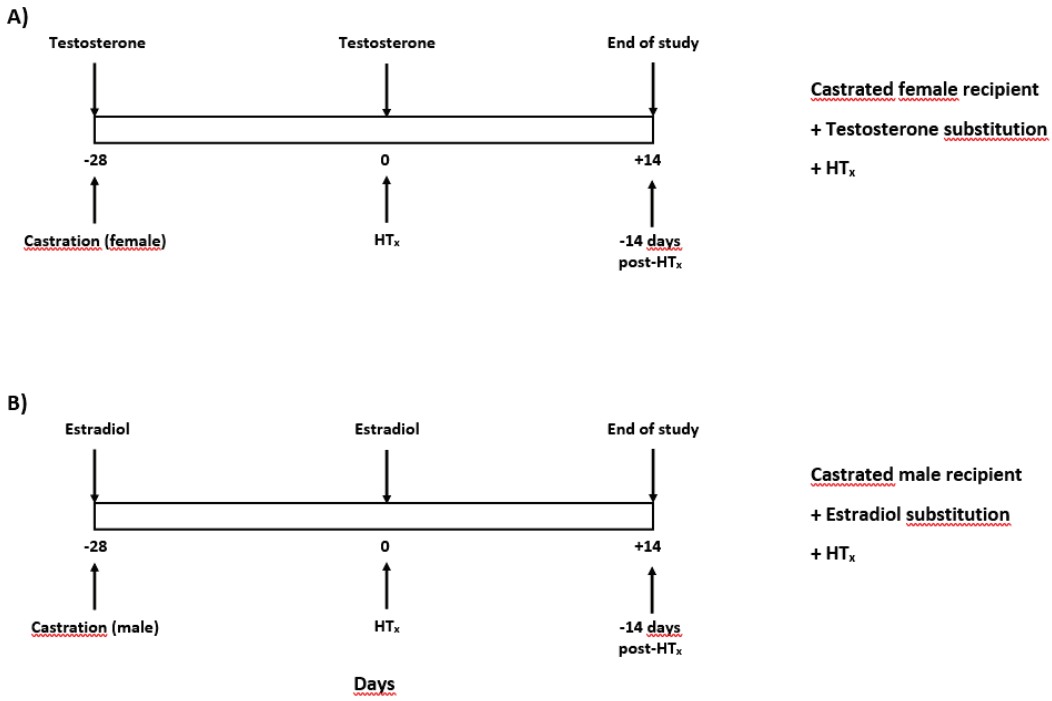

Fig. 2. An outline of the set of experiments evaluating the course of cardiac atrophy after heterotopic heart transplantation (HT_X) in Lewis rats after castration and exposed to substitution of steroid hormones of the opposite sex.

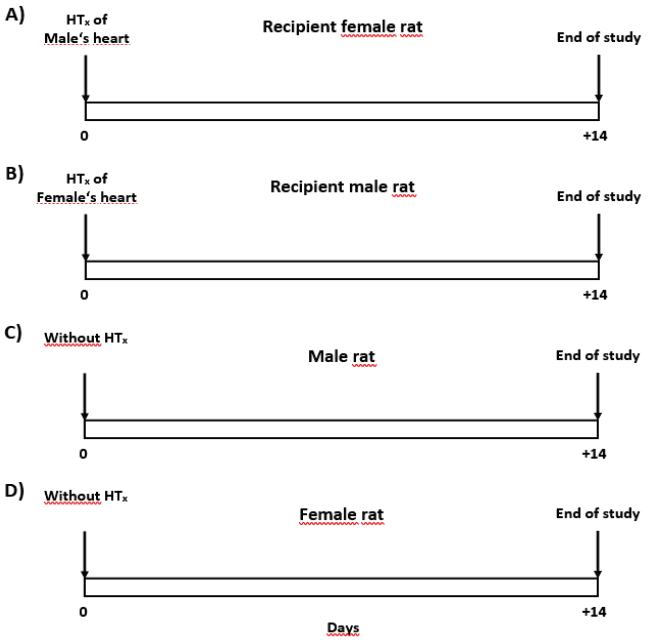

Fig. 3. An outline of the set of experiments evaluating the course of cardiac atrophy after heterotopic heart transplantation (HT_X) to Lewis rat recipients of the sex opposite to that of the donor´s heart.

Results

Fig. 4 summarizes results of our initial studies which evaluated the effectiveness of hormonal substitution by steroid hormones of the opposite sex. Within 7 days after castration of male as well as female Lewis rats, a profound decrease was observed in plasma testosterone, down to levels 6 to 8 times lower than observed in intact female Lewis rats. Testosterone substitution in castrated female Lewis rats increased within 2 days plasma testosterone to levels measured in intact male Lewis rats (Fig. 4A). Likewise, castration of female as well as male Lewis rats markedly decreased plasma estradiol levels, and estradiol substitution in castrated male Lewis rats increased plasma estradiol levels to levels that are comparable to those observed in intact female Lewis rats (Fig. 4B). Again, this occurred within 2 days after initiating of the substitution.

Table 1 collects the absolute values of whole HW, LVW, and RVW of the native and transplanted hearts 14 days after HT_X. The values for the native heart, either in the chest of the castrated recipient or in the chest of the intact control counterpart served as basal values (100 %) for evaluation of the process of cardiac atrophy and, when the same sex was compared, there were no significant differences between the values in the chest of castrated animals versus those in the chest of intact animals. As expected, the weight of the native hearts in the chest of the castrated recipients or in the chest of their intact control counterparts was significantly lower in female Lewis than in male Lewis rats.

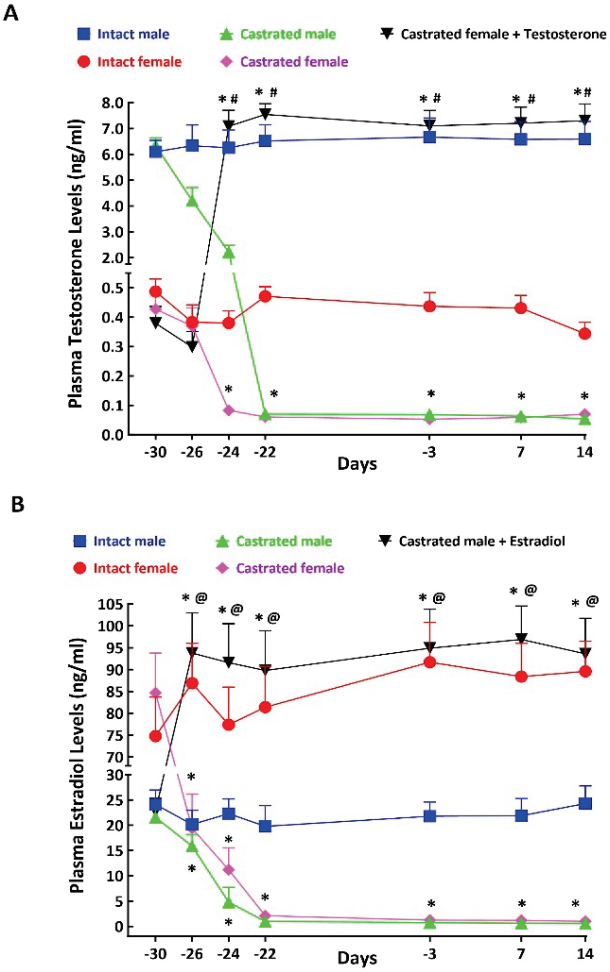

Fig. 4. Plasma levels of testosterone (**A**) and estradiol (**B**) in the series of studies evaluating the effectiveness of hormonal substitution with steroid hormones of the opposite sex. *P<0.05 compared with basal values (day -30, i.e. before castration). #P<0.05 compared with the values of intact female at the same time point. @ P<0.05 compared with the values of intact male at the same time point.

Table 1. The weight of the native (recipient) heart and the transplanted (donor heart and of the individual hert 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.

	Parameter					
	HW (mg) (native)	HW (mg) (HT_x)	LVW (mg) (native)	LVW (mg) (HT_x)	RVW (mg) (native)	RVW (mg) (HT_x)
Castrated male recipient + estradiol substitution + HT_x of male donor´s heart	1024 ± 27	568 ± 16	691 ± 18	372 ± 16	182 ± 8	134 ± 5
Intact recipient female + HT_x of male donor´s heart		570 ± 18		374 ± 15		139 ± 8
Intact male without HT_x	1031 ± 29		692 ± 19		181 ± 7	
Castrated female recipient + testosterone supplementation + HT_x of female donor´s heart	762 ± 20*	657 ± 9*	509 ± 8*	447 ± 4*	137 ± 8*	123 ± 7*
Intact recipient male + HT_x of female donor´s heart		660 ± 10*		449 ± 6*		125 ± 5*
Intact female without HT_x	768 ± 23*		512 ± 10*		138 ± 6*	

Values are means ± SEM. HT_x, heterotopic heart transplantation; HW, whole heart weight; LVW, left ventricule weight; RVW, right ventricule weight. . * P<0.05 vs. male (i.e. effects sex differences on the parameter measured).

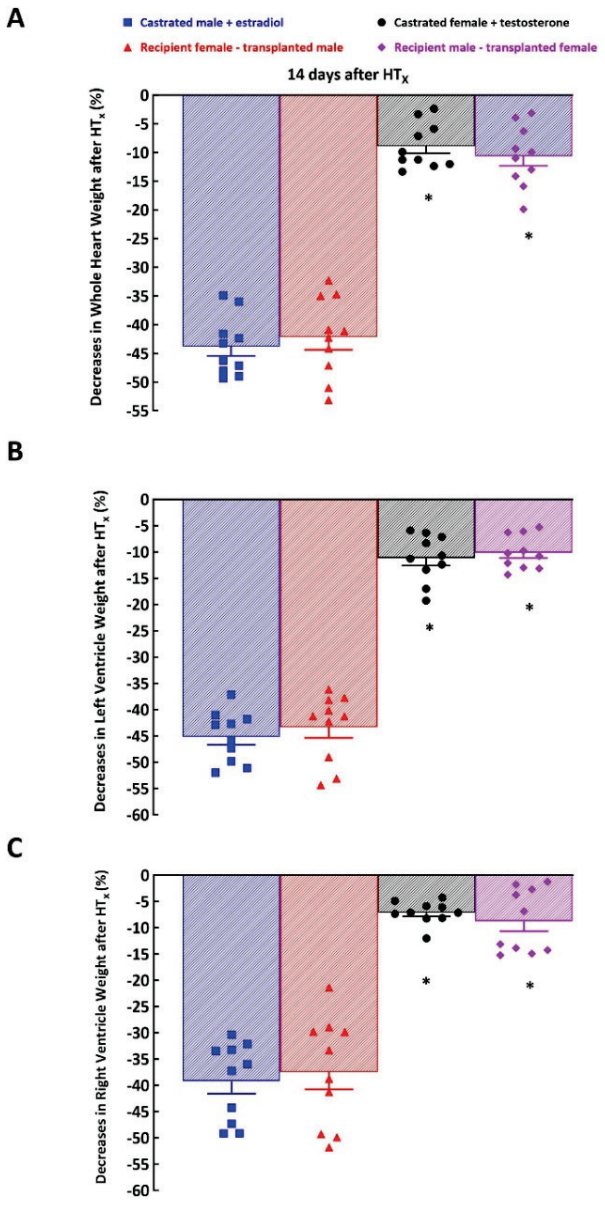

Fig. 5. Effects of either substitution of steroid hormones of the opposite sex in castrated animals or heterotopic heart transplantation (HT_x) into the recipient of the sex opposite to that of the donor´s heart on the course of cardiac atrophy in response to mechanical heart unloading induced by (HT_x) 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.

As shown in Fig. 5A, 14 days after mechanical unloading induced by HT_X, female rats under both experimental set-ups (i.e., when the female heart was transplanted to either castrated female recipients exposed to testosterone substitution or to intact male recipients) displayed significantly lower decreases in whole HW when compared to the decreases observed in male rats exposed to the same experimental arrangements (-9 ± 1 and -11 + 1 vs. -44 ± 2 and -42 ± 2 %, p<0.05 in all cases). The dynamics of changes in LVW and RVW after HT_X were quite similar as those in whole HW (Figs 5B and 5C).

Fig. 6A shows whole HW values of the native heart normalized to tibia length (TL). This is the standard approach to assess cardiac mass in groups of animals with significant differences in BW. The HW/TL ratio values show that male rats (in both experimental arrangements) have higher mass of the native hearts when compared to those of female rats (again, in both experimental arrangements). As shown in Fig. 6B, 14 days after HT_X, the decreases in whole HW were more significant in male rats after mechanical unloading induced by HT_X, making this ratio significantly higher in the female than in male rats (again in both experimental arrangements).

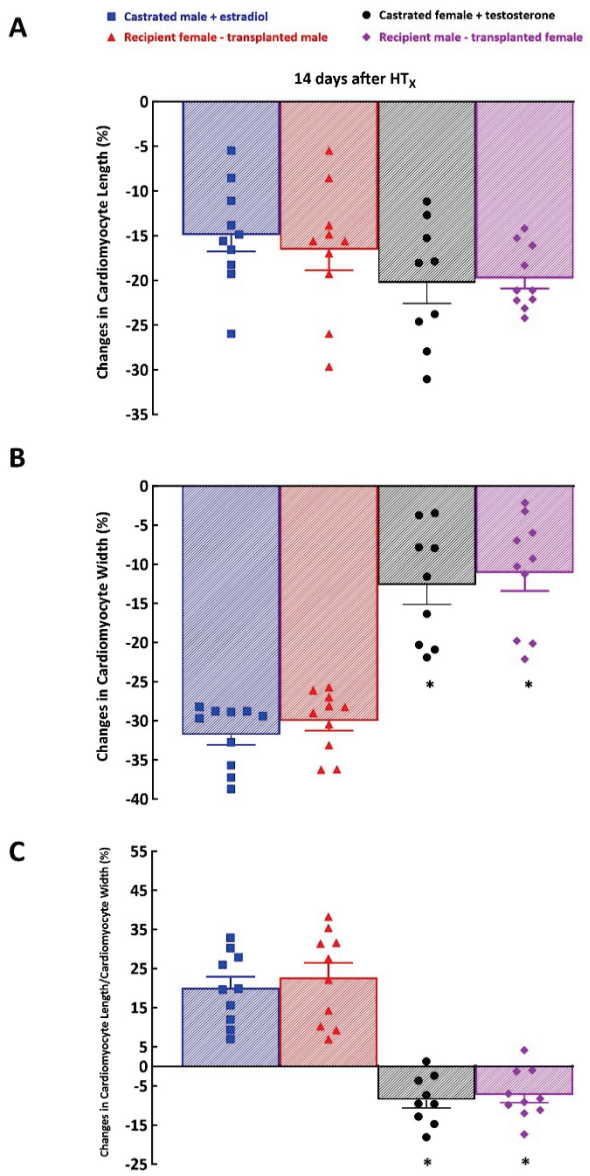

Fig. 6. Effects of either substitution of steroid hormones of the opposite sex in castrated animals or heterotopic heart transplantation (HT_x) into the recipient of the sex opposite to that of the donor´s heart on the course of the whole heart weight to tibia length ratio over 14 days after heterotopic heart transplantation (HT_x) in male and female Lewis rats. (**A**) values in native (i.e. orthotropic) heart, (**B**) values in transplanted (i.e. heterotopic) heart. *P<0.05 compared with male animals.

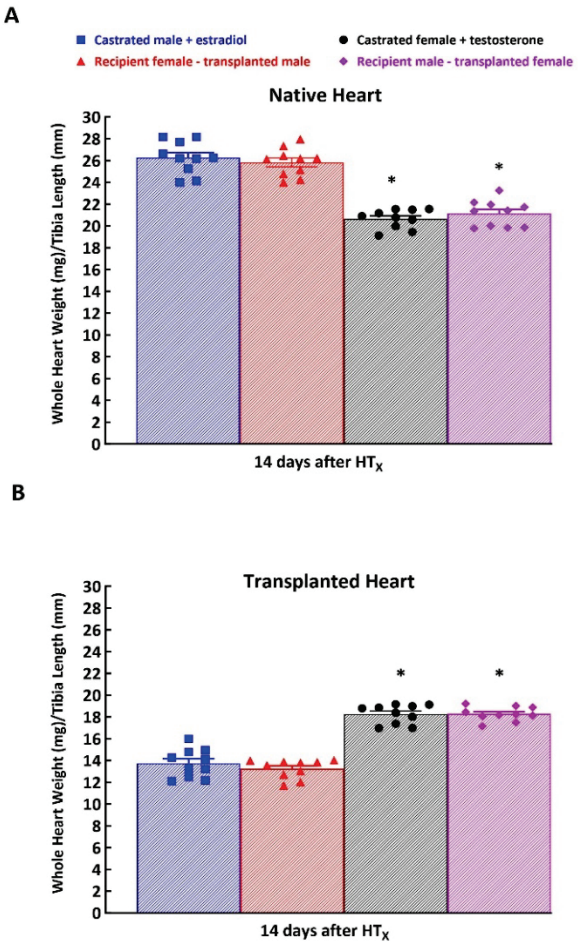

Fig. 7. Effects of either substitution of steroid hormones of the opposite sex in castrated animals or heterotopic heart transplantation (HT_x) into the recipient of the sex opposite to that of the donor´s heart on the course of myocyte size change in the left ventricle in response to mechanical heart 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.

Table 2 summarizes the absolute values of the parameters of the myocyte size, specifically cardiomyocyte length (CL), cardiomyocyte width (CW), and the ratio of CL to CW in the native and transplanted hearts measured 14 days after HT_X. As can be inferred from the data, there were no significant differences between the values of the CL, CW and the CL to CW ratio in the chest of castrated animals when compared to the values measured in the chest of intact animals, similarly in male and female rats.

As shown in Fig. 7A, in all experimental groups, mechanical unloading induced by HT_X caused similar decreases in CL. In contrast, in female rats under both experimental arrangements (with female heart transplanted to either castrated female recipient exposed to testosterone substitution or to intact male recipients), HT_X caused significantly smaller decreases in CW when compared to male rats exposed to the same experimental conditions (-13 ± 2 and -11 + 1 vs. -32 ± 2 and -31 ± 1 %, $p<0.05$ in all cases) (Fig. 7B). As shown in Fig. 7C, under both experimental arrangements, the augmented decreases in CW in male rats when compared to those of female rats caused significant increases in CL to CW ratios in male rats when compared to female rats (+20 ± 1 and +23 + 2 vs. -7 ± 1 and -7 ± 1 %, $p<0.05$ in all cases).

Table 2. Cardiomyocyte length and cardiomyocyte width of the native (recipient) heart and the transplanted (donor) 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 (mg) (HT_x)
Castrated male recipient + estradiol substitution + HT_x of male donor´s heart	124.1 ± 1.7	104.9 ± 2.6	16.3 ± 0.4	10.6 ± 0.6	7.6 ± 0.2	9.9 ± 0.5
Intact recipient female + HT_x of male donor´s heart		103.4 ± 2.8		11.1 ± 0.5		9.4 ± 0.4
Intact male without HT_x	122.4 ± 1.9		16.6 ± 0.5		7.5 ± 0.2	
Castrated female recipient + testosterone supplementation + HT_x of female donor´s heart	119.8 ± 2.1	97.2 ± 2.1	16.2 ± 0.4	13.2 ± 0.3*	7.5 ± 0.3	7.3 ± 0.3*
Intact recipient male + HT_x of female donor´s heart		98.1 ± 1.8		13.4 ± 0.2*		7.4 ± 0.3*
Intact female without HT_x	120.1 ± 1.9		16.1 ± 0.5		7.5 ± 0.3	

Values are means ± SEM. Cl, cardiomyocyte length; CW, cardiomyocyte width; HT_x, heterotopic heart transplantation. * $P<0.05$ vs. Male (i.e. effects sex differences on the parameter measured).

Discussion

The critically important finding of the present study is that attenuation of unloading-induced cardiac atrophy in female rats when compared to male rats does not depend on the actions of sex hormones. If this were the case, the differences in the development of cardiac atrophy would exist in the hormones' presence and disappear after their removal [38].

This conclusion is based on our present results showing that neither castration of female rats combined with substitution of testosterone nor HT_X of female heart into the male recipient worsened the process of unloading-induced cardiac atrophy in female rats. Furthermore, since we showed that gonadectomy did not augment the process of unloading-induced cardiac atrophy induced by HT_X in female rats [36], we can conclude that attenuation of unloading-induced cardiac atrophy in female rats is not related to protective effects of estradiol or due to the absence of testosterone at concentrations observed in their male counterparts.

Of particular interest is our finding that testosterone substitution in castrated female rats did not exhibit any detrimental effects on the course of cardiac atrophy. Moreover, our present results show that neither castration of male rats combined with substitution of estradiol nor HT_X of male heart into the female recipient attenuated the process of unloading-induced cardiac atrophy in male rats. In this context, our recent study showed that gonadectomy did not diminish the process of unloading-induced cardiac atrophy after HT_X [36]. Thus, we can conclude that the augmented unloading-induced cardiac atrophy in male rats is not associated with detrimental actions of testosterone or the lack of protective effects of estradiol.

Our pertinent recent and present findings at the whole organ level are corroborated at the cardiomyocyte level. Again, this was seen under native conditions and also under conditions of each of the three experimental arrangements: in animals after gonadectomy, animals after gonadectomy with substitution of steroid hormone of the opposite sex, and in the experiment with the heart transplanted to the recipient of the opposite sex.

Based on the results of our present and our most recent study [36], we are convinced that sex-linked differences in the process of unloading-induced cardiac atrophy, specifically augmentation of this process in male animals, cannot be simply ascribed to the deleterious actions of testosterone in males or to the protective effects of estradiol in females. We are aware that this conclusion refers only to "activational" effects of sex hormones as described above and not to "organizational" effects of sex hormones, which persist long after sex hormones have been removed from circulation. It is believed that the latter effects are dominantly mediated by changes in DNA structure and chromatin remodeling i.e. by different epigenetic modification of DNA [34,38]. Our recent and present study aimed, first, to establish whether sex-linked differences in the development of unloading-induced cardiac atrophy do exist and, if this was the case, if they simply depend on environmental conditions dependent on plasma concentrations of sex hormones. Therefore, we cannot provide any further information regarding the mechanisms responsible for the

sex-linked differences in the course of the atrophy. Nevertheless, it is now recognized that numerous genetic as well as epigenetic mechanisms play a major role in mediating the sex differences [34,38] so that future studies are needed to address this very complex issue.

Our studies of animals which were either post gonadectomy on steroid hormone substitution of the opposite sex, or after HT_X to the recipient of the opposite sex, have unexpectedly generated noteworthy information. Transgender medical care is an area that is rapidly expanding, despite the fact that it is disregarded in the health care system. However, as reported from the Los Angeles Williams Institute of the University of California, about 0.6 % of the US adult population is transgender (https://williamsinstitute.law.ucla.edu/publications/trans-adults-united-states). Transgender individuals often undergo so called "gender-affirming hormone therapy" (GAHT), intended to elicit secondary sexual character-istics of the affirmed gender. It will be noticed that in those individuals, sex steroids are administered at large doses that are highly unphysiological [42]. There is growing concern about the long-term adverse effects of GATH, because recent evidence suggests that such treatment is associated with an increased risk of cardiovascular disease [42-45]. However, because of only limited information about the effects of cross-sex steroid application on the cardiovascular system, a call for more clinical as well as basic research in the field of transgender medicine is rising [42]. Our present findings clearly show that un-physiological concentrations of steroid hormones incompatible with the biological sex does not have any beneficial or detrimental effect on the course of unloading-induced cardiac atrophy, which suggests that the course of cardiac atrophy after HT_x is dominantly dependent on inherent properties of the donor´s (i.e. transplanted) heart. Our data suggests that in transgender individuals the course of cardiac atrophy after long-term LVAD would correspond to the actual biological sex and would not be altered by GAHT. However, it is important to admit that our present studies are relatively short-term and that in the long-term perspective GAHT could exhibit some detrimental effects either at the cellular or functional level. If our preclinical results are confirmed by clinical studies, this would imply that not only in women but also in so called "trans men" (sometimes identified as of male gender but assigned female sex at birth) undergoing GAHT, LVAD-induced cardiac atrophy would be attenuated when compared to men as well as so called "trans women" (someone who identifies as of female gender but was assigned male sex at birth).

Sex-linked differences in function of the human healthy heart and in HT: general considerations

It is known that after puberty LV mass shows sex-dependent differences, as the male cardiomyocyte undergoes greater hypertrophy than in the female. Moreover, the female heart is generally smaller but in proportion to smaller body size. There are also sex-linked differences in electrophysiology of the heart, e.g. in the action potential of parameters and ionic currents of cardiomyocytes. Specifically, the duration of action potential is longer in female cardiomyocytes, consistent with clinical observation that women have longer rate-corrected QT interval. Furthermore, there are multiple sex-linked differences in the Ca^{2+} handling in cardiomyocytes, leading to the differences in excitation-contracting coupling and contractility of cardiomyocytes. The sex differences in the healthy heart were recently appraised in a comprehensive review [46].

Regarding the sex-linked differences in HT_x, it should first be noted that in human medicine, the terms of sex and gender are often used interchangeably, which results in some misleading interpretations. *Sex* refers to an organism´s biological sex, while *gender* refers to roles associated with the sex of an individual and with social and cultural roles of the person. There are important sex-linked as well as gender-based differences of approach in the HT_x program. This issue is excellently handled in a recent review [47] and therefore only the most important sex- and gender-based differences are highlighted here. Sex-specific considerations in HT_X are divided into "Pre-Transplant", "Peri-Transplant" and "Post-Transplant" categories.

In the "Pre-Transplant" phase, women are often referred with a considerable delay to an advanced HF specialist, which results in a lower rate of LVAD implantation indications and translating to an unconscious bias against women in candidate selection for HT_x. These are gender-related rather than sex-related differences. In addition, in this phase women exhibit higher risk of allosensitization, which is related to a history of pregnancy. Moreover, women also display markedly higher challenges in gaining access to temporary mechanical circulatory support, and are exposed to a higher risk of complications. Such divergences can be described as typically sex-linked.

In the "Peri-Transplant" phase the major issue was the finding that if a female donor was used for a male recipient, reduced post-HT_x survival was reported. However, such sex-mismatching proved a misconception: it was later found that if appropriately sized female donor heart was used (similar size heart from a male donor was employed for male recipient), the outcomes observed would be equally good.

In the "Post-Transplant" phase, higher rates and severity of rejection in women is observed. On the contrary, women exhibit lower cardiac allograft vasculopathy and a lower rate of malignancies and cancer-related deaths. However, it should be noticed that the males undergoing HT_x tend to be older and have higher prevalence of obesity, diabetes, dyslipidemia and, in particular, a history of tobacco use. Therefore, it seems that in such cases we have to deal with gender-related rather than sex-related differences. Thus, it is apparent that sex-specific characteristics must be considered before application of HT_x in humans. Evidently, experimental, as well as clinical studies are needed to evaluate sex-linked differences in the treatment of advanced HF.

It is worthwhile to emphasize again that sex-linked differences in the cardiovascular system and cardiovascular diseases should not be solely ascribed to actions of estradiol and testosterone and the activity level of their receptors. Alternative mechanisms, such as the influence of other sex hormones, sex chromosome-linked genes, incomplete X-chromosomal gene inactivation, histone and DNA modifications, interactions of sex hormones with different neurohormonal systems in different organs must be taken into consideration. This extremely complex issue of sex differences in cardiovascular system was recently appraised in some comprehensive reviews [34,38,48].

Conclusions, merits and perspectives

Our present data clearly shows that the enhanced development of unloading-induced cardiac atrophy in female as compared to male rats does not depend on activational actions of sex hormones. Future studies are needed to evaluate more thoroughly the mechanisms responsible for the sex-linked differences in the course of unloading-induced cardiac atrophy. The information obtained in our recent and present preclinical studies should be considered in prospective clinical studies designed to explore the development of cardiac atrophy in response to LVAD-induced mechanical unloading and in the search for new therapeutic measures against this process.

Author contribution

All authors have read and approved the final version of the manuscript. D.M.K., I.M., and M.P. performed all experiments related to heterotopic heart transplantation and evaluation of plasma concentrations of sex hormones. P.K. performed all histological evaluations. P.Š. performed all sham operations and gonadectomy and participated in the preparation of tissue samples for histological evaluation. Z.V. performed all RIA analyses of steroid hormones. J.S., L.Č., and I.N. were responsible for conceptualization, data evaluation, writing of the original draft, and review of the manuscript. I.N. is also responsible for funding acquisition.

Conflict of Interest

There is no conflict of interest.

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