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Sex differences in cardiac graft recovery and pathophysiologic changes in a rat model of donation after circulatory death

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Biological sex affects cardiac tolerance to ischemia and reperfusion, but little is known about sex differences in heart transplantation with donation after circulatory death (DCD). Using a rat model, we characterized differences between sexes both in the donor, during simulated DCD, and in the procured heart, to evaluate recovery upon reperfusion. Adult male, female and ovariectomized Wistar rats underwent simulated DCD with 22 min of warm, *in-situ* ischemia. Hearts were then explanted and maintained in cold static storage for 30 min. Isolated hearts then underwent normothermic, oxygenated reperfusion for 60 min with left ventricular loading. *In-situ* characteristics, as well as ventricular function, coronary flows and metabolic post-ischemic recovery were assessed. Post-ischemic ventricular recovery was significantly higher in female compared to male and ovariectomized hearts. Furthermore, several *in-situ* circulating factors, including free fatty acids and potassium, were significantly lower in females compared with males. Greater post-ischemic ventricular function in female vs. male and ovariectomized hearts following DCD indicates that sex affects tolerance to DCD conditions and that female sex hormones contribute to cardioprotection. Greater understanding of sex-specific tolerance to DCD conditions may serve as a basis to better define precise mechanisms of cardiac injury and thereby contribute to optimizing clinical protocols.

Keywords Sex as a biological variable (SABV), Heart transplantation, Donation after circulatory death (DCD), Cardiac ischemia/reperfusion injury, *Ex-situ*/*ex-vivo* heart perfusion

Abbreviations

DCD	Donation after circulatory death
FFA	Free fatty acids
FWIT	Functional warm ischemic time
H-FABP	Heart-type fatty acid binding protein
LV	Left ventricular
OVX	Ovariectomized rats

Heart donation after circulatory death (DCD) is used to overcome organ shortage, holding the potential to increase transplantation activity by 26–48%[1,2] and provides recipient outcomes comparable to conventional brain-death donations[3–5].

Despite the excellent clinical results with DCD in heart transplantation, room for optimization of clinical protocols remains[6–8]. Maastricht Type III donors are typical in DCD heart transplantation and, after withdrawal of life sustaining therapy, hearts are subjected to potentially damaging conditions, including hypoxia. In porcine models of DCD, increases in circulating lactate and potassium (K^+) and a catecholamine surge have been reported[9–11]. These conditions in the DCD donor, including a period of warm cardiac ischemia, lead to profound changes in cardiac metabolism and may influence graft quality[12].

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It is recognized that sex differences in cardiac ischemic resilience and metabolism exist. A recent report demonstrated the influence of sex on global myocardial ischemia tolerance and mitochondrial function in a rat model of circulatory death[13]. In fact, DCD hearts from female rats present relatively less damaged interfibrillar mitochondria[13], recognized as the primary source of energy to the contractile apparatus[14,15]. Furthermore, it was shown that mitochondria from female hearts produce less reactive oxygen species compared to mitochondria from males, thereby contributing to improved mitochondrial calcium retention capacity[13]. Following myocardial infarction, reproductive-age female rats demonstrated smaller infarct sizes and higher ventricular function than age-matched males[16], in line with higher ischemic tolerance in reproductive-age females compared to males[16].

Female sex hormones play a key role in the improved ischemic tolerance of female hearts. Indeed, estrogen can provide cardioprotection by preserving the vasculature, improving excitation-contraction coupling and inhibiting mitochondrial death[17]. Correspondingly, in mouse models of ischemia/reperfusion injury, the administration of estrogen to males partially salvaged cardiac recovery, while ovariectomy in females led to worse cardiac recovery[16]. Less is known about the effects of progesterone in cardiac ischemic tolerance; however, its administration to female rats improved the recovery in terms of infarct size, oxidative stress, inflammation and arrhythmias, whereas it did not change recovery in hearts from males or ovariectomized (OVX) rats[18].

In the context of conventional heart transplantation after brain death, female donor hearts were considered less favorable, especially when transplanted into male recipients, primarily due to presumed sex mismatch[19,20]. However, recent studies, including in DCD heart transplantation, have shown that sex mismatch per se does not impair graft recovery or survival[21,22]. Instead, worse outcomes are mainly explained by donor-recipient size mismatch[21,22]. When size matching is achieved, graft recovery and survival are comparable regardless of donor or recipient sex[21,22]. Overall, females are underrepresented in clinical studies and a better understanding of sex-dependent differences should permit optimization of clinical protocols and application of precision therapies at graft procurement, during *ex-situ* heart perfusion and potentially post-transplantation[23].

Therefore, we aimed to investigate sex differences cardiac graft recovery following simulated DCD with a period of *in-situ* ischemia, measured as ventricular and metabolic function, as well as coronary flow profiles, using a rat model of DCD. Furthermore, we characterized the pathophysiological changes that occur in the donor and may affect the cardiac recovery after warm, *in-situ* ischemia and reperfusion in this model. To investigate the role of both sex and female sex hormones, male, female and OVX rats were included in the study.

Results
Baseline characteristics
A total of 20 rats (6 males, 7 females and 7 OVX) were included in this study (exclusive of additional rats used for estrus stage evaluation). Sample size and baseline characteristics are shown in Table 1. Body weight, heart weight and systolic arterial pressure were lower in female and OVX rats than in males (body weight: both $p < 0.001$; heart weight: females $p < 0.001$, OVX $p = 0.002$; systolic pressure: females $p = 0.009$, OVX $p < 0.001$). In addition, female body weight was lower compared to OVX ($p < 0.001$). Heart rate was similar among groups. Progesterone and estradiol levels in the rats were significantly higher in females compared to both males and OVX ($p < 0.001$ for all).

Improved myocardial recovery in females
Post-ischemic myocardial recovery is presented in Fig. 1. Left ventricular (LV) work (Fig. 1a), maximum contraction rate (Fig. 1b) and maximum relaxation rate (Fig. 1c) were significantly greater in females compared to males and OVX ($p < 0.001$ for all). Cardiac output (Fig. 1d) was similar among groups.

Release of the cell death markers troponin I, heart-type fatty acid binding protein (H-FABP) and myoglobin was similar among groups (Figure S1); however, H-FABP (Fig. 1e, $p = 0.035$, Spearman's rho $= -0.579$) and myoglobin (Fig. 1f, $p = 0.023$, Spearman's rho $= -0.632$) showed negative correlations with cardiac output.

	Males	Females	OVX
Sample size	6	7	7
Body weight (g)	400 [391–411]	234 [220–256] * $p < 0.001$ \$ $p < 0.001$	282 [278–305] * $p < 0.001$
Heart weight (g)	1.84 [1.68–2.05]	1.18 [1.15–1.22] * $p < 0.001$	1.35 [1.28–1.47] * $p = 0.002$
Systolic arterial pressure (mmHg)	112 [99–118]	91 [82–101] * $p = 0.009$	80 [74–88] * $p = 0.001$
Pulse pressure [mmHg]	14 [12–18]	21 [7–23]	9 [3–15]
Heart rate (beats * min^{-1})	252 [224–392]	226 [202–285]	215 [193–226]
Progesterone (ng * mL^{-1})	2.26 [1.32–4.01]	27.35 [14.14–40.24] * $p < 0.001$ \$ $p < 0.001$	3.57 [2.77–5.70]
Estradiol (pg * mL^{-1})	1.61 [1.40–2.76]	6.26 [5.71–9.25] * $p < 0.001$ \$ $p < 0.001$	1.93 [1.36–2.23]

Table 1. Baseline characteristics. *: vs. males, \$: vs. OVX, OVX: ovariectomized rats; $n = 6$–7/group.
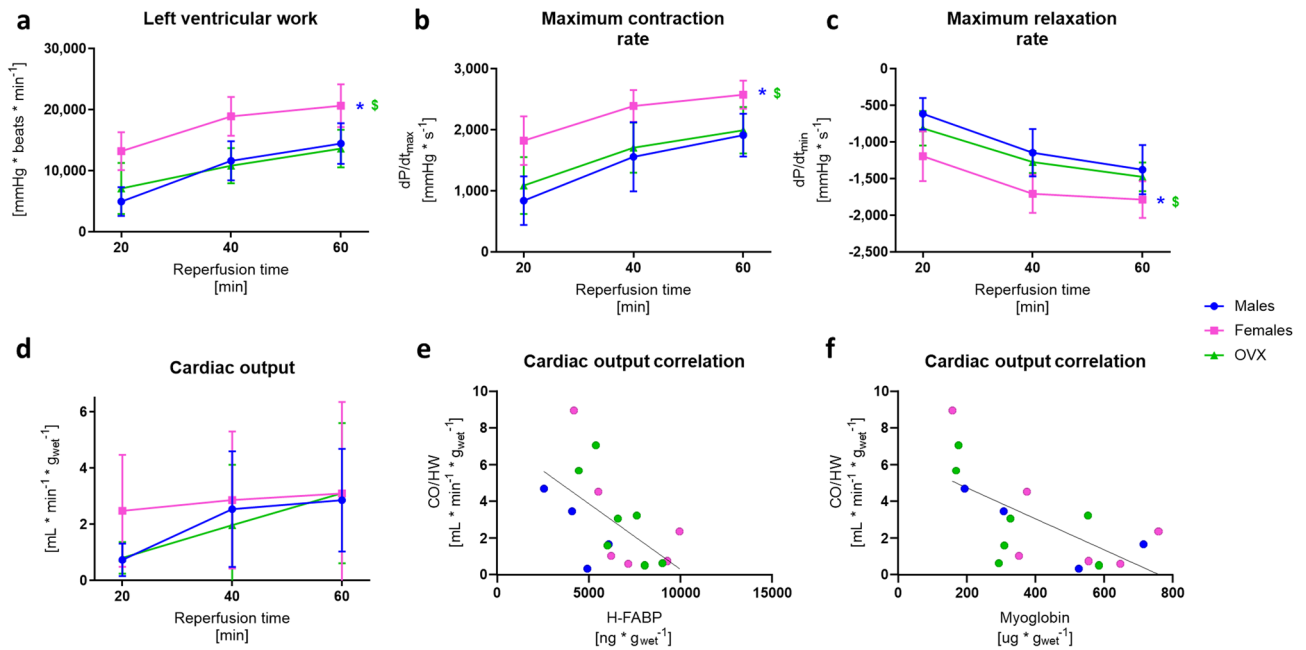
Fig. 1. Ventricular function and markers of cell death during reperfusion with *ex-situ* heart perfusion. (**a**) Left ventricular work reported as mean ± SD, (**b**) maximum contraction rate reported as mean ± SD, (**c**) maximum relaxation rate reported as mean ± SD, (**d**) cardiac output reported as mean ± SD, (**e**) correlation between the release of heart-type fatty acid binding protein over 60 min of reperfusion with cardiac output, $p = 0.035$, Spearman's rho = − 0.579, (**f**) correlation between the release of myoglobin over 60 min of reperfusion with cardiac output. $p = 0.023$, Spearman's rho = − 0.632, *$p < 0.001$ females vs. males, \$ $p < 0.001$ females vs. OVX. Symbols indicate statistical significance based on linear regression analysis over 20 to 60 min reperfusion. CO: cardiac output; dP/dt$_{max}$: maximum first derivative of left ventricular pressure; dP/dt$_{min}$: minimum first derivative of left ventricular pressure; HW: heart weight; H-FABP: heart-type fatty acid binding protein; $n = 5–7$/group.

Active hyperemia is higher in males
Coronary flows were evaluated as reactive hyperemia (coronary flow during the first minutes of aerobic reperfusion) or active hyperemia (change in coronary flow with increasing cardiac workload triggered by switching from unloaded to loaded perfusion modes). Reactive hyperemia (Fig. 2a) was not different between sexes, whereas active hyperemia (Fig. 2b) was significantly lower in females and OVX compared to males ($p = 0.034$, $p = 0.033$; respectively). No difference among experimental groups was observed for coronary flows (Fig. 2c) or tissue edema after 60 min reperfusion (Fig. 2d).

Improved oxygen efficiency in females
Oxygen consumption was greater in males and females compared to OVX hearts (Fig. 3a; $p = 0.002$ for males, $p = 0.043$ for females). Oxygen efficiency was significantly higher in female hearts compared to male hearts (Fig. 3b, $p = 0.002$). Release of cytochrome c (Fig. 3c), an indicator of mitochondrial damage, release of 8-hydroxy-2′-deoxyguanosine (Fig. 3d), an indicator of oxidative stress, and calcium content in cardiac tissue after reperfusion (Fig. 3e) were not different between groups. In addition, the net increase in perfusate lactate (Fig. 3f) was significantly lower in females than in OVX ($p = 0.009$).

Estrus cycle stage correlates with myocardial recovery
The females were classified for estrus cycle stage according to vaginal swab findings (Figure S2). Although we have a small number of female rats in each stage, the females in estrus seemed to present the lowest ventricular function (Figure S3). In addition, estrus cycle stage correlated with cardiac output (Figure S3e, $p = 0.017$, Spearman's rho = − 0.766) and oxygen efficiency (Figure S3f; $p = 0.036$, Spearman's rho = − 0.715).

Sex differences *in-situ*
Overall, the progression of biochemical changes after the start of functional warm ischemic time (FWIT) in all groups followed a pattern similar to that previously reported in male rat models[24] and in porcine models[9–11] (Fig. 4). Blood glucose concentration (Fig. 4e), circulating free fatty acids (FFA, Fig. 4f) and blood potassium concentration (Fig. 4g) were significantly lower in females compared to males at baseline ($p = 0.014$, $p < 0.001$, $p = 0.022$; respectively) and FWIT ($p = 0.002$, $p = 0.002$; $p = 0.032$; respectively). Blood glucose and potassium were also significantly lower in females compared to OVX at baseline ($p = 0.008$, $p = 0.003$; respectively). Blood glucose concentration and FFA were significantly lower in OVX compared to males at FWIT ($p = 0.049$, $p < 0.001$; respectively) and FFA were also significantly lower in OVX compared to males at baseline ($p < 0.001$).
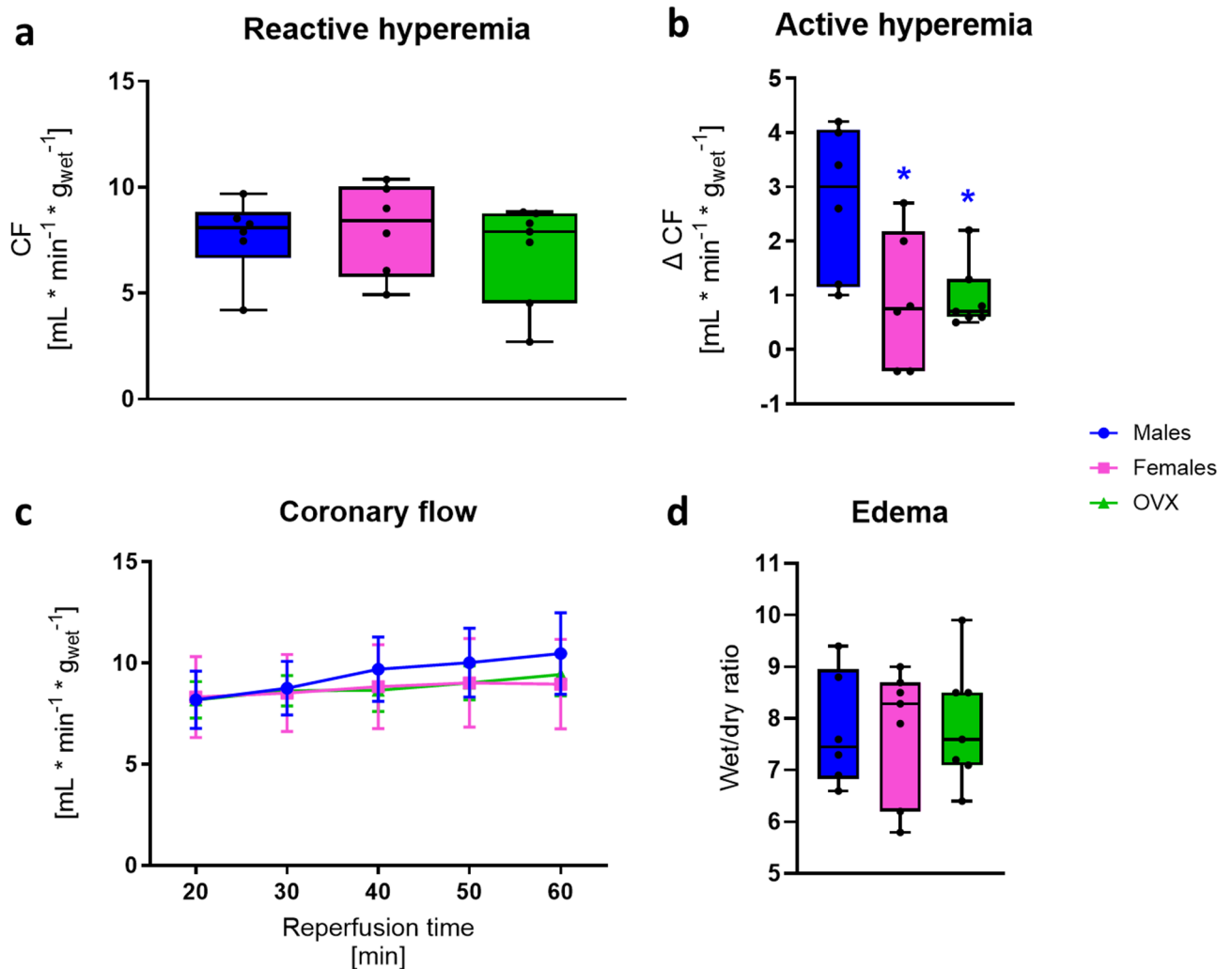
Fig. 2. Coronary flow and edema during reperfusion with *ex-situ* heart perfusion. (**a**) Reactive hyperemia measured as the mean coronary flow during early unloaded reperfusion (3 min, 5 min and 10 min of reperfusion), (**b**) active hyperemia measured as increase in coronary flow between unloaded and loaded conditions, *$p = 0.034$ females vs. males, * $p = 0.033$ OVX vs. males, (**c**) coronary flow during loaded mode reported as mean ± SD, (**d**) edema formation. CF: coronary flow normalized by heart weight, $n = 6$–7/group.

Blood calcium concentration (Fig. 4h) was elevated at end-ischemia, but was not significantly different among experimental groups. It was significantly lower in female and OVX hearts at baseline and FWIT compared with end-ischemia ($p < 0.001$ for OVX and females at baseline, $p = 0.036$ for females at FWIT). Adrenalin levels (Fig. 4i) were not different between sexes; however, noradrenalin levels (Fig. 4j) were increased in females and OVX compared to males at end-ischemia ($p = 0.023$, $p = 0.025$; respectively).

Discussion

In this study, we aimed to investigate sex differences in post-ischemic recovery of ventricular and metabolic function, as well as coronary flow profiles and to characterize the corresponding pathophysiological changes in the DCD donor that may affect cardiac graft quality. To investigate the role of female sex hormones, males, females and OVX rats were included. We report improved myocardial recovery in hearts from reproductive-age female rats compared with hearts from male and OVX rats, after simulation of DCD with *in-situ* warm ischemia, indicating a cardioprotective role for female sex hormones. Furthermore, metabolic recovery, determined by oxygen efficiency, was greater in females compared with males, supporting the concept of improved preservation of mitochondrial function. We also characterized sex-specific differences *in-situ* during simulation of the DCD protocol. In general, high, stable calcium levels in males with higher circulating FFA, glucose, and potassium in males versus females, may all contribute to the decreased myocardial recovery in male versus female hearts. Although the present model does not simulate extended-criteria donors, it provides valuable mechanistic insight into sex-related differences in the myocardial response to ischemia and reperfusion after circulatory death. These findings contribute to our understanding and shed light on potential mechanisms underlying sex differences in cardiac graft recovery following simulated DCD. These findings should also help with the advancement of
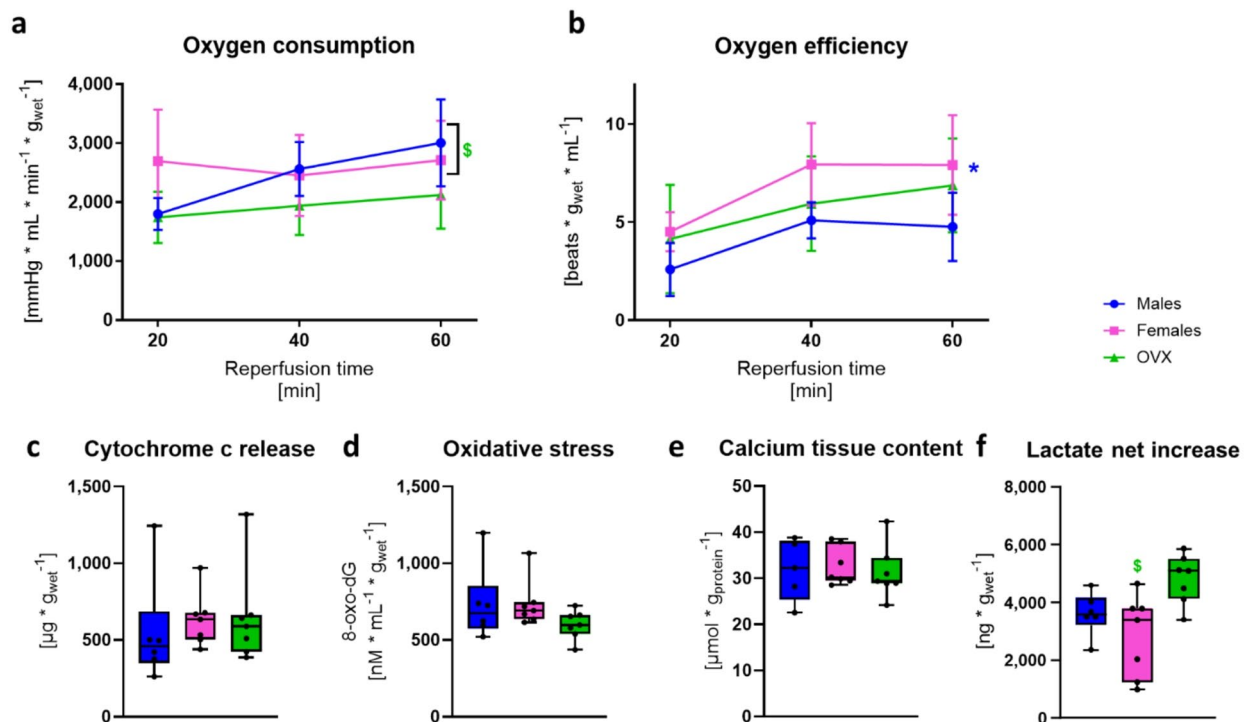
Fig. 3. Metabolic recovery and oxidative stress. (**a**) Oxygen consumption during the loaded reperfusion reported as mean ± SD, $ $p = 0.043$ females vs. OVX, $ $p = 0.002$ males vs. OVX, (**b**) oxygen efficiency (calculated as the quotient of LV work and oxygen consumption) reported as mean ± SD, * $p = 0.002$ females vs. males, (**c**) cytochrome c release over 60 min of reperfusion, (**d**) oxidative stress over 60 min of reperfusion, (**e**) calcium content in cardiac tissue after 60 min of reperfusion, (**f**) net increase in lactate over 60 min of reperfusion, $ $p = 0.009$ females vs. OVX, symbols on panels (a) and (b) indicate statistical significance based on linear regression analysis over 20 to 60min reperfusion. 8-oxo-dG: 8-hydroxy-2′-deoxyguanosine, $n = 6$–7/ group.

precision medicine approaches towards optimizing current clinical protocols, especially with respect to therapies that could be applied during *ex-vivo* perfusion and post-transplantation These approaches are relevant not only for heart transplantation, but also for other DCD organs/ transplantation approaches.

We report that, during reperfusion, recovery of ventricular function was increased in reproductive-age females compared to males and OVX rats. The inclusion of OVX rats permits investigation of the influence of female sex hormones on myocardial recovery, which is of particular relevance as female sex hormones are reported to be cardioprotective[16] and may help to preserve ventricular function in the context of DCD heart transplantation. In parallel with improved ventricular function, we also report significantly improved oxygen efficiency in female hearts compared to males. Improved oxygen efficiency in females compared to males is consistent with improved mitochondrial function. In line with our study, Chen et al. showed that myocardial recovery is higher in female compared to male rats following a DCD protocol and reported less damage in interfibrillar mitochondria from female compared with male DCD hearts[13]. Relatively less damaged interfibrillar mitochondria would be expected to improve energy supply in female DCD hearts during reperfusion which, in turn, should contribute to a better recovery of ventricular function in females compared with males after ischemia[13]. Previous studies also reported that post-ischemic recovery of isolated working hearts from female rats was lower compared to males when hearts were perfused with a solution containing glucose and fatty acids, a composition similar to what we used[25,26]. However, these studies were not performed in the context of DCD heart transplantation. Overall, we report higher post-ischemic cardiac and mitochondrial function in females compared to males and OVX, indicating metabolic differences between the groups, potentially mediated by sex hormones.

To our knowledge, our study is the first to study sex differences in coronary flows in DCD hearts. We did not see differences in reactive hyperemia. Given that reactive hyperemia is largely driven by rapid vasodilators such as nitric oxide (NO) and adenosine[27,28] in response to ischemia[29], comparable responses in males, females and OVX imply that neither sex, nor sex hormones, significantly enhance or impair this type of flow response under these experimental conditions. On the other hand, we demonstrate that, while female and OVX rats exhibited similar active hyperemia, male rats demonstrated a greater active hyperemia, in line with a previous report in humans[30]. The significantly elevated active hyperemia in males suggests a sex-dependent difference in vascular adaptation. Testosterone is reported to be a vasodilatory hormone[31] and potentially mediates the increased hyperemic response observed in male rats compared to female and OVX. Similar rates of coronary flow per gram of tissue were generally measured across all groups throughout the reperfusion period, indicating
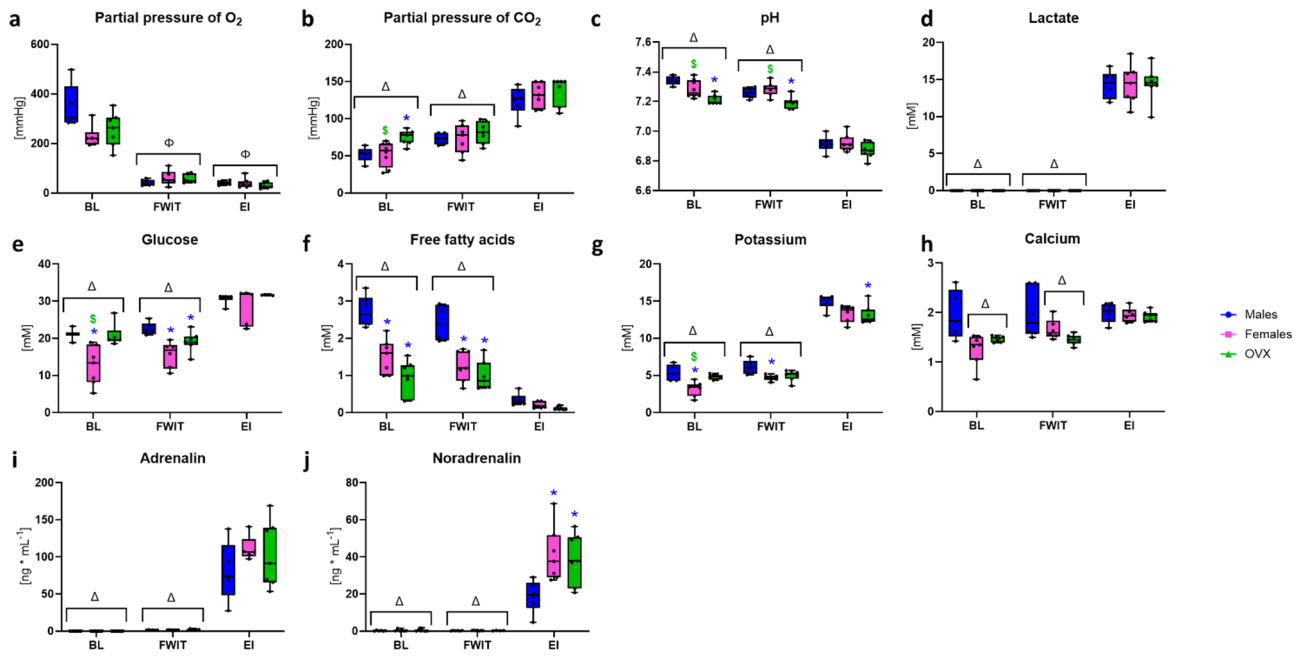
Fig. 4. *In-situ* blood measurements during the DCD protocol at baseline (BL), start of functional warm ischemia (FWIT) and at the end of ischemia (EI). (**a**) partial pressure of oxygen, Φ $p < 0.001$ vs. respective baseline, (**b**) partial pressure of carbon dioxide, Δ $p < 0.001$ vs. respective end ischemia, $ $p = 0.025$ females vs. OVX at baseline, * $p = 0.011$ OVX vs. males at baseline, (**c**) pH, Δ $p < 0.001$ vs. respective end ischemia, $ $p = 0.041$ females vs. OVX at baseline, * $p < 0.001$ OVX vs. males at baseline, $ $p = 0.007$ females vs. OVX at FWIT, * $p = 0.036$ OVX vs. males at FWIT, (**d**) lactate concentration, Δ $p < 0.001$ vs. respective end ischemia, (**e**) glucose concentration, Δ $p < 0.001$ vs. respective end ischemia, * $p = 0.014$ females vs. males at baseline, $ $p = 0.008$ females vs. OVX at baseline, * $p = 0.002$ females vs. males at FWIT, * $p = 0.049$ OVX vs. males at FWIT, (**f**) free fatty acid concentration, Δ $p < 0.001$ vs. respective end ischemia, * $p < 0.001$ females vs. males at baseline, * $p < 0.001$ OVX vs. males at baseline, * $p = 0.002$ females vs. males at FWIT, * $p < 0.001$ OVX vs. males at FWIT. (**g**) potassium concentration, Δ $p < 0.001$ vs. respective end ischemia, * $p = 0.022$ females vs. males at baseline, $ $p = 0.003$ females vs. OVX at baseline, * $p = 0.032$ females vs. males at FWIT, * $p = 0.042$ OVX vs. males at end ischemia. (**h**) calcium concentration, Δ $p < 0.001$ vs. respective end ischemia for OVX and females at baseline, Δ $p = 0.036$ for females at FWIT, (**i**) adrenalin concentration, Δ $p < 0.001$ vs. respective end ischemia, (**j**) noradrenalin concentration, Δ $p < 0.001$ vs. respective end ischemia, * $p = 0.023$ females vs. males at end ischemia, * $p = 0.025$ OVX vs. males at end ischemia, BL: baseline; FWIT: functional warm ischemic time; EI: end ischemia; $n = 6–7$/group.

similar rates of oxygen delivery. As oxygen efficiency was significantly higher in female vs. male hearts, which corresponds with a previous report of higher mitochondrial function in females[13], these findings suggest that male hearts are less effective in adapting to meet the energy requirements under post-DCD loaded conditions compared to female hearts.

To our knowledge, we are the first to investigate sex differences in circulating FFA levels. Already at baseline, FFA concentrations were high in males and females compared to physiologic values[32]. We have previously reported that FFA levels increase in males in both rat[24] and in pig models[33] of DCD. Increases in circulating FFA are recognized under other conditions of acute myocardial ischemia, and are elevated in response to both heparin[34] and increased circulating catecholamines[35]. It has been shown that females are less sensitive to lipolytic stimulation[36] and our results of a limited increase in FFA in female and OVX rats compared with male rats support this concept. Lastly, in our study, catecholamines are low at BL and FWIT, which is an indication that the high FFA levels in our rats were initially due to heparin administration and not the catecholamine surge. Heparin is crucial in our model to prevent coagulation in the donor following circulatory arrest. Interestingly, we observed that ovariectomized animals did not always resemble males, as would be expected if ovarian hormones were the sole determinant of the observed differences. For example, noradrenaline levels did not follow this pattern. This suggests that additional mechanisms may be involved beyond circulating female sex hormones. It should be noted that ovariectomized rats remain biologically female, and sex-specific factors such as chromosomal influences, epigenetic regulation, or intrinsic differences in autonomic control and receptor expression may contribute to these findings. Therefore, our results highlight the importance of distinguishing between the effects of circulating sex hormones and those of biological sex per se, both of which may influence cardiac responses in the setting of ischemia–reperfusion injury.

Hormonal fluctuations in the estrus cycle have long been considered responsible for variability in female rat models. However, over the past 10 years, studies have begun to challenge this hypothesis. For example, similar to our study, a meta-analysis of 293 publications found no difference in variability between male and female

rats[37,38]. Furthermore, variability between male and female rats across physiological phenotypes related to the heart, lungs, blood vessels, blood, and kidneys was not different[39]. However, it is important to note that some studies have reported estrous-cycle-specific effects on certain behaviors[40]. In fact, Ramíres-Hernàndez et al. have shown that infarct size is increased for rats in estrus stage after myocardial infarction[41]. Our study aligns with these findings, as estrus appears to correspond with lower recovery of ventricular function and estrus stage significantly correlates with myocardial recovery, despite a small number of females in each stage.

In the clinical setting, it has been described that cardiac grafts from females are often unfavorable, especially when implanted in male recipients[19,20]. However, in our study using a DCD protocol, we showed that female hearts retain higher ventricular function than males following DCD simulation, and OVX rats recovered similarly to the males. It has been shown that estrogen inhibits the opening of connexin43 in the mitochondria, and thereby preserves mitochondrial function after ischemia[42]. It has also been demonstrated that, if estrogen was given to male rats during reperfusion, LV function was increased and infarct size was decreased following ischemia[42]. Estrogen in female rats might be a key player in the observed improved recovery, as estrogen is reported to activate multiple salvaging pathways in the ischemia/reperfusion setting, such as ERK or MAPK which lead to increased mitochondrial gene expression and biogenesis, higher respiration rates and ATP production and the production of antioxidant enzymes. Furthermore, estrogen can also lead to a decrease in mitochondrial apoptotic pathway, cytochrome c release, reactive oxygen species production, calcium accumulation[43] and an improved mitochondrial quality control[44–47]. In addition, progesterone receptors can act as transcription factors or activate PKC, Akt or MAPK pathways to stimulate cell survival or proliferation[48]. On the other hand, testosterone is recognized to decrease cardiac function[49] which might be a relevant mechanism for the observed functional decrease observed in males compared to females and OVX.

This study possesses some limitations. One limitation is that, due to the small blood volume in rats, the hearts were not perfused with blood but with a crystalloid solution. This condition may not fully replicate the clinical situation, thereby limiting the direct translatability of our findings to human transplantation. With the model used, only short-term myocardial recovery was assessed, transplantation studies with longer term recovery are now warranted. The model used is a controlled DCD donor model without prior brain injury. While this approach does not mimic extended-criteria or clinically marginal donors, it was chosen to minimize biological variability and to ensure reproducible ischemia-reperfusion conditions. The reperfusion period was not designed to reproduce the entire clinical setting (e.g., ex-vivo perfusion using Organ Care System technology), but to allow precise control of reperfusion conditions. This strategy enabled the investigation of intrinsic sex-related differences in tolerance to DCD-induced conditions including a clinically relevant period of in-situ ischemia, while limiting variability and the number of animals required, in accordance with 3R principles. Future studies including aged or comorbid donors and clinically relevant graft preservation approaches will be essential to translate these findings to the clinical context. In order to investigate the influence of sex hormones on myocardial recovery, OVX rats were included with age-matched males and females; however, it is important to acknowledge that sex differences are not solely driven by circulating sex hormones.

In conclusion, we have investigated sex-specific differences in post-ischemic ventricular function, coronary flows and metabolism, and have characterized *in-situ* pathophysiologic changes in the donor using a rat model of DCD. The post-DCD recovery of myocardial function was higher in hearts from females compared to males and OVX. As OVX rats showed a pattern of ventricular function recovery that was more similar to males than females, our findings support the concept that female sex hormones contribute to improved tolerance to ischemia/reperfusion injury in the context of DCD heart transplantation. Importantly, we also demonstrate greater post-DCD oxygen efficiency in females vs. males, consistent with a previous report of better preserved mitochondrial function in female hearts. Furthermore, pathophysiological changes in the donor, that are present even before heart procurement in the context of DCD - such as elevated circulating FFA, glucose and potassium, as well as a tendency for increased calcium levels in males vs. females - may contribute to the reduced myocardial recovery subsequently observed following reperfusion and ventricular loading. In addition to helping identify precision cardiac therapies to be applied during *ex-situ* heart perfusion or post-transplantation that could optimize clinical DCD protocols, this study also describes a rat model of DCD and provides a basis for future studies that further investigate sex as a biological variable in DCD transplantation.

Materials and methods
Ethics statement
All experiments were reviewed and approved by the Swiss animal welfare authorities and the ethics committee for Animal Experimentation, Bern, Switzerland (BE13/2022). All procedures involving animals were performed in accordance with relevant national guidelines and institutional regulations for animal welfare, and are reported in compliance with the ARRIVE guidelines.

Animals
Male, female and OVX Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France) were housed in groups with a 12 h light-dark cycle at a controlled room temperature and with unlimited access to food and water. Rats underwent ovariectomy or sham surgery at approximately 6 weeks of age and were used for experiments at 10 to 11 weeks of age, representing young-adult human DCD heart donors with a mature cardiac metabolism[50,51].

Experimental protocol
Rats were anesthetized with an intraperitoneal injection of ketamine (78 mg/kg for males; 65 mg/kg for females and OVX; Narkatan, Vetoquinol AG, Bern, Switzerland), acepromazine (1.2 mg/kg for males; 1.0 mg/kg for females and OVX; Prequillan, Bologna, Italy) and xylazine (7.2 mg/kg for males; 6.0 mg/kg for females and OVX; Xylapan, Vetoquinol AG, Bern, Switzerland). Simulation of DCD was performed as previously reported[24]
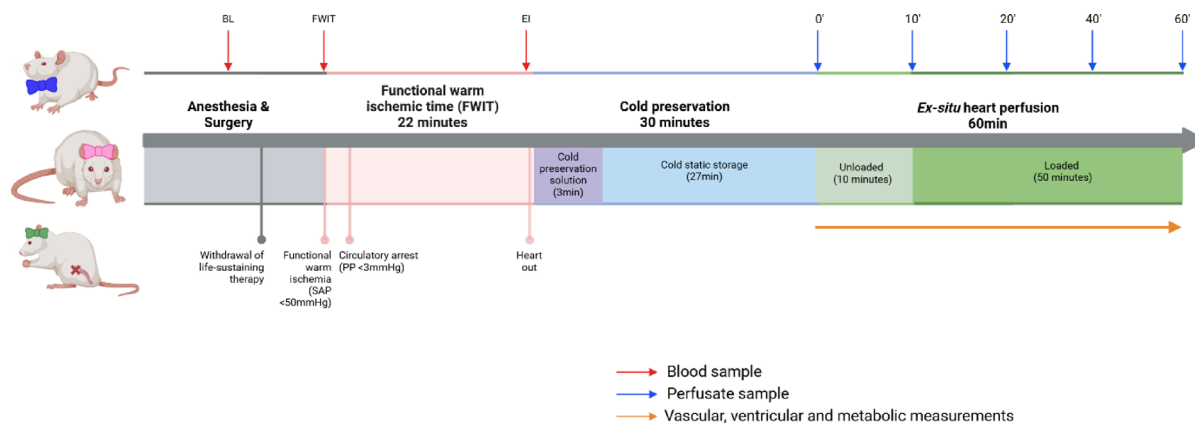
Fig. 5. Experimental protocol. Anesthetized males, females and ovariectomized females underwent circulatory arrest and 22 min of functional warm ischemia. Afterwards, hearts were procured and mounted on an *ex-situ* heart perfusion system, flushed for 3 min with a cold preservation solution and stored for another 27 min at 4 °C. Hearts were then perfused for 10 min in the unloaded mode, followed by 50 min with left ventricular loading. *In-situ* blood samples (red arrows) were taken after cannulation of the carotid artery (baseline-BL), at start of functional warm ischemia time (FWIT) and after excising the heart (end ischemia-EI). Perfusate samples (blue arrows) were taken at 0, 10, 20, 40 and 60 min of *ex-situ* perfusion. Coronary flow, ventricular and metabolic recovery (orange arrow) were evaluated throughout the loaded perfusion. BL: baseline; FWIT: functional warm ischemic time; EI: end ischemia; SAP: systolic arterial pressure; PP: pulse pressure.

and is represented in Fig. 5. Briefly, following loss of the pedal withdrawal reflex, the right carotid artery was cannulated to monitor blood pressure and 320U of heparin were injected in order to avoid blood coagulation. Withdrawal of life sustaining therapy was simulated by transecting the diaphragm. FWIT was considered to start when the systolic arterial pressure reached 50mmHg. Circulatory arrest was declared when the pulse pressure was 3mmHg. Temperature was monitored during the entire protocol and rats were maintained at 37 °C with a heat lamp. Once 22 min of ischemia had elapsed, hearts were quickly explanted, mounted on an *ex-situ* perfusion system and flushed with a cold preservation solution (St. Thomas II cardioplegia supplemented with glyceryl trinitrate and erythropoietin) for 3 min at a constant pressure of 60 mmHg. No fixed volume was predetermined; the total perfusate volume ranged from 16.5 mL to 28 mL, depending on the pressure-driven flow during this period. The cardioplegia flush was followed by 27 min of cold static storage at 4 °C. 22 min of *in-situ*, warm ischemia[52] was chosen based on a previous study to generate an intermediate myocardial recovery and 30 min of cold ischemia[3,4] was chosen to simulate the time needed in a clinical setting to prepare the heart for perfusion. Isolated hearts were then aerobically perfused and warmed to 30 °C for 1 min in a non-recirculating unloaded mode and 9 min in a recirculating unloaded mode at 37 °C. LV loading was then performed for 50 min. Hearts were perfused with modified Krebs-Henseleit buffer supplemented with 0,45 mM bovine serum albumin, 0.5 mM lactate and 0.4 mM palmitate. Preload (left atrial) pressure was set to 11.5 mmHg and afterload (aortic) pressure was set to 60 mmHg.

In-situ blood samples were taken after cannulation of the carotid artery (baseline), at the start of FWIT and after explanting the heart (end-ischemia). Preload and coronary effluent perfusate samples were taken at 0, 10, 20, 40 and 60 min of ex-situ perfusion. After 60 min reperfusion, hearts were snap frozen and stored at − 80 °C for further analysis.

Myocardial recovery evaluation
LV pressure was measured using a micro-tip pressure catheter (Millar; Houston, USA) placed via the mitral valve. Perfusate flow was measured using flow probes (Transonic Systems Inc.; Ithaca, USA) placed in pre- and after- load lines. Cardiac output and coronary flow were normalized by heart weight. Reactive hyperemia was measured as the mean absolute value of coronary flow normalized by heart weight during early unloaded reperfusion (1, 3 and 5 min of reperfusion). Active hyperemia was measured as the difference in absolute values of coronary flow normalized by heart weight between unloaded and loaded conditions (respectively 10 and 40 min of reperfusion). Data were continuously recorded using a PowerLab data acquisition system and analyzed with LabChart 7 software (ADInstruments; Oxford, UK).

Blood gas analysis (in-situ blood and perfusate samples)
Partial oxygen and carbon dioxide pressure, electrolytes, pH, glucose and lactate were measured using a Cobas b 123 blood gas analyzer (Roche; Basel, Switzerland). For glucose measurements, values that exceeded the 30 mM maximal measurement threshold were set to 30 mM for analysis.

Biochemical measurements
Biochemical measurements are described in Supplemental Material.

Estrus cycle determination

The estrus cycle was determined by vaginal lavage, the detailed methods are described in Supplementary Material. An additional series of female rats that underwent the same experimental protocol was included in order to increase the number of female rats for the determination of estrus cycle and investigation of its effects on myocardial recovery.

Statistics

All results are expressed as median and interquartile range. Statistical analyses were performed with GraphPad Prism version 9 (GraphPad Software, La Jolla, CA). To limit variability, we applied a predefined set of exclusion criteria (technical issues, coronary flow cut-off above 13 mL/min/g, warm ischemic temperature cut-offs below 35 °C or above 37 °C, and cannulation time exceeding 2 min). The Tukey test was used to identify outliers, which were then removed from the study. For variables with repeated measures, a linear regression analysis was used to evaluate differences between groups. One-way ANOVAs were to determine differences among variables measured at one timepoint only, while two-way ANOVAs were used to determine differences among sex groups for variables measured at different timepoints *in-situ*. Following ANOVA tests, a Fishers Least Significant Difference test was used to determine differences between experimental groups. Correlations were analyzed using the Spearman's rank correlation test.

To control for type I error, all reported *p*-values were subsequently adjusted for multiple comparisons using a modified Bonferroni procedure[53]. Corrected *p*-values were reported, and statistical significance was considered at $p < 0.05$.

Data availability

The data that supports the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

All authors contributed to the work and agreed with the content of this manuscript. MA, MS and SL obtained funding. AC, SG, DL, AH, MA, ME and AS performed experiments and collected data. AC, SG, MS, MA and SL performed the data analysis and interpretation. AC, SG wrote the manuscript; all authors revised it and gave feedback.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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