

## **Influence of gender on ischemia-reperfusion injury in lungs in an animal model**

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**Short title:**

Influence of gender on ischemia-reperfusion injury in lungs

## **Summary**

As with other organ transplants even lung transplantation raises the question of the possibility of the influence of gender on ischemia-reperfusion injury. This is a current topic especially for increasingly utilized method of lung transplantation from a non-heart-beating donors, where reperfusion preceded by a period of warm and cold ischemia with subsequent treatment options for lung graft reperfusion.

For measurements we used our laboratory previously created and validated animal model for ex vivo lung transplantation. As with other organ systems of our monitoring resulted protective effect of female sex on ischemia reperfusion lung injury. In two of the three parameters that were monitored, we found a significant difference. In females, a higher oxygen transfer ability after reperfusion was manifested as well as lower perfusion pressure (vascular compliance). Conversely, weight gain (the development of pulmonary edema) in males was not significant difference from the females.

These conclusions could cause further studies leading to influence the selection of appropriate donor grafts.

## **Keywords:**

Lung Transplantation/ex vivo

Reperfusion Injury/prevention & control,

Gender difference

Animal model

## 1. Introduction

As in other areas of medicine, in the field of lung transplantation there is the question of the influence of gender on the survival and thriving of the graft. Lung ischemia-reperfusion (IR) injury leading to lung failure is an important clinical problem mainly during transplantation from non-heart-beating donors (NHBDs), allowing the treatment of the lung outside the body. However, the number of patients that could benefit from lung transplantation greatly exceeds the number of donors available (Cypel et al. 2009). Despite refinements in lung preservation and improvements in surgical techniques and perioperative care, ischemia-reperfusion-induced lung injury remains a significant cause of early morbidity and mortality after lung transplantation (De Perrot et al. 2003). Therefore, as in the previous study (Mrazkova et al. 2015), we focused our experiment on the period of IR injury in NHBDs and we studied the sex differences during this period.

The presumption of a different tissue response to IR injury in males and females is based on previous studies in other organ systems (Harada et al. 2001; Müller et al. 2000; Murphy et al. 2007) and on the gender difference in lung diseases based on Carey MA et al. 2007. It is well known that numerous health problems are affected by gender (Ostadal et al. 2009).

Free radical mediated injury occurring in ischemic tissue after reperfusion is a major cause of damage in lung transplantation. Hypoxia accelerates the production of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide (De Perrot et al. 2003; Kennedy et al. 1998).

Bae and Zang 2005 and Wang et al. 2006 observed gender differences in cardioprotection in some animal studies suggesting that, compared to males, intact pre-menopausal females (without exogenous estrogen treatment) have reduced IR injury. Barp et al. 2010 indicate that protection against oxidative damage is provided by enzymatic and non-enzymatic antioxidant defenses.

The aim of our study is to confirm or refute the protective influence of the female sex, for ischemia-reperfusion injury in lung tissue, as is the case with the protective influence against ischemia-reperfusion injury in other organ systems.

## 2. Materials and methods

Two groups of adult male Wistar rats and two groups of female Wistar rats were subjected to experiments in accordance with the European Community and NIH guidelines for using experimental animals. Two to three month old male or female Wistar rats weighing  $250 \pm 30$  g were divided into four groups with 6 animals per group.

Two experimental groups (M-male, F-female, Tab. 1) passed the protocol for non-heart-beating-donors (NHBD). The experimental procedure was based on the protocol used in previous studies in our laboratory (Hodyc et al. 2008; Mrazkova et al. 2015).

The experimental animals were anesthetized with thiopental (100 mg/kg BW IP), placed in the supine position and secured to the table. 250 IU of heparin was administered into the liver under direct vision. After 5 min, the animals were euthanized by an overdose of sodium thiopental (250 mg/kg BW IP) and we cannulated their tracheae. Then followed a period of warm ischemia, when these animals were kept at room temperature without ventilation for 90 min.

After a period of warm ischemia, we opened the thorax and cannulated both pleural cavities – one cannula for inflow and a second one served for the outflow of the perfusion solution (PSS with 5% of Ficoll) which was at a temperature of 12°C and was circulated through the chest of the experimental animal. It was placed in a cooling chamber at a temperature of 12 °C for 60 min (cold ischemia). During the period of cold ischemia, the animals were not ventilated.

Then the lungs were isolated and perfused using the standard method described previously (Hodyc et al. 2008; Mrazkova et al. 2015). The tracheal cannula was connected to the ventilator and the lungs were ventilated by positive pressure (inspiratory pressure 12 cm H<sub>2</sub>O, expiratory pressure 3 cm H<sub>2</sub>O) with a gas mixture containing 21% O<sub>2</sub> + 5% CO<sub>2</sub> balanced with N<sub>2</sub> (Herget et Chovanec 2010). The pulmonary artery and the left atrium were cannulated. Using a peristaltic pump, the lungs were perfused in a constant flow mode (4 ml per 100 g body weight per min) with a saline solution containing 4% Ficoll and meclofenamate (6-10 mol/liter). The lungs were suspended by the trachea on the force transducer in a heated (38°C) humidified chamber and allowed to stabilize for 15 min before the measurement of lung function. The outflow from the left atrial cannula was recirculated into the perfusate reservoir, and the outflow pressure was set to -2 mm Hg.

We assessed the pulmonary functions for 120 min after the 15 minutes' stabilization from the beginning of the perfusion. The pulmonary perfusion pressure was monitored via a transducer connected to the inflow cannula (PowerLab, ADI Instruments). The lung weight gain was measured continually by a force transducer on which the isolated lungs were suspended. After a 20 min equilibration period, the first measurement of oxygen transport ability was performed. We took two samples of perfusion solution – an arterial sample from the inflow and a venous sample from the outflow cannula. The partial pressure of oxygen was measured immediately after sampling (ABL™ 5, Radiometer Medical A/S, Copenhagen, Denmark). Since we used isolated lungs, it was necessary to desaturate the inflow perfusate artificially by bubbling the perfusate in the reservoir with 5% CO<sub>2</sub> + 95% N<sub>2</sub> for 5 min. Thus, the increase in oxygen partial pressure between the inflow and outflow cannula ( $\Delta pO_2$ ) was a measure of the ability of the lung to transport oxygen from the alveoli into the perfusate.

After a 10-min recovery period, the reactivity to angiotensin II (0.4 µg) and to acute hypoxic challenge (0% O<sub>2</sub> + 5% CO<sub>2</sub> + 74% N<sub>2</sub>) was tested in two cycles as a test of lung viability (Herget and Chovanec 2010). One lung of group M and one of group F were discarded due to poor response to hypoxic challenge.

In time periods of 30, 60, 90 and 120 min from the beginning of the perfusion, the lung transport ability for oxygen was measured in the same way as described above and in these time intervals the values of perfusion pressure and lung weight gain were recorded.

Control groups of animals (Cm-controls male, Cf-controls female, Tab. 1) were anesthetized and the isolation of the lung was performed in the same manner as described above. However, the perfusion of the lungs started immediately after isolation, thus the lungs did not undergo warm and cold ischemia.

Free radical concentration, biological antioxidant potential (PAT test), was measured in perfusate samples taken from the venous reservoir after 90 min of warm ischemia PAT test (FRAS-II, Italy) (Bertuglia and Giusti 2005). The test measures antioxidant potential by chromatic change of ammonium thiocyanate. The intensity of this will be directly proportional to the plasma ability to reduce the ferric ions to ferrous ion.

#### *Statistical analysis*

For the statistical evaluation we used ANOVA for repeated measures, Fisher's PLSD test and Games/Howell post hoc test as appropriate. Mean values  $\pm$  SEM are presented.

### 3. Results

The experimental and control groups contained the lungs which survived until the end of all functional assessments. All preparations retained significant vasoconstrictor responses to angiotensin II and acute ventilator hypoxia.

#### 3.1. Oxygen transport ability

Measuring oxygen transport ability using the arterial-venous difference in oxygen partial pressure, we found significantly higher oxygenation capability in female rat lungs (F) compared to the male rat lungs (M) –  $p < 0.05$ . The best oxygen transport ability, especially at the beginning of perfusion, was observed in the control female group (Cf), and not only was it significantly higher than the experimental groups (F, M), but also than the control male group (Cm) (Fig. 2).

#### 3.2. Pulmonary perfusion pressure

In constant flow perfusion, the pulmonary arterial pressure is determined by the resistance of pulmonary vasculature to perfusion flow. It was significantly ( $p < 0.05$ ) lower in the female experimental lungs (F) than in the male rat group (M) (Fig. 3).

We did not notice a significant difference between the control groups (Cf, Cm) or between the experimental (F, M) and control groups (Cf, Cm).

#### 3.3. Weight gain

Weight gain during the experiment was caused by the development of pulmonary edema. There was no significant difference between experimental male and female groups (F, M). The only significant difference was observed between the experimental male and control male group (M, Cm) –  $p < 0.05$  (Fig. 4).

#### 3.4. ROS measurement

The values of biological antioxidant potential (PAT) indicated higher oxygen radical stress in male rat preparations that went through the period of warm ischemia. PAT test in perfusate of experimental female rats was  $350 \pm 30$  and in experimental male rats  $225 \pm 35$  IU Carr.

#### 4. Discussion

From our study, which focused on the influence of gender difference on IR injury of lung after cold and warm ischemia in NHBD protocol, we made three conclusions:

- There is a gender-linked difference in tolerance of warm and cold ischemia based on the significantly lower ability to transport O<sub>2</sub> in the lungs of experimental males versus females.
- The female sex also has a significant protective effect on the development of vascular resistance to perfusion flow after IR injury, which resulted in significantly higher perfusion pressure in experimental male animals.
- In our observation of weight gain as proof of the development of pulmonary edema, we did not see significant sexual dimorphism in experimental groups.

A main cause of damage occurring in ischemic lung tissue after reperfusion is the release of free radicals (Kennedy et al. 1989; McCord 1985; Hoidal et al. 1998). During reperfusion, the sudden availability of oxygen to act as a cofactor allows xanthine oxidase to convert purine substrate accumulated during ischemia to uric acid, with superoxide anions and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) forming as byproducts (Kennedy et al. 1989). Hydroxyl radicals are potent oxidants that cause tissue injury by initiating lipid peroxidation of cell membranes and oxidative inactivation of critical cell proteins (Kennedy et al. 1989; Cohen et al. 1985).

Why are the females more resistant to IR lung injury? The increased resistance to ROS has been shown by several studies on laboratory animals (for a review see Carrey et al. 2007). Firstly, estrogens exert antioxidant activity. This has not been fully elucidated and involves multiple mechanisms (Harada et al. 2001). Numerous studies in vitro have shown that estrogens have the potential to affect the redox chemistry of transition metals and, in particular, iron and copper. By this mechanism, estrogens are thought to inhibit peroxidation of membrane lipids and lipoproteins (Lacort et al. 1995; Ruiz-Larrea et al. 1995). The down regulation of inducible NO synthase may be another factor (Kawachi et al. 2000; Yu et al. 2006). The link to the increase in pulmonary capillary permeability and to the difference in perfusion pressure is evident. Estrogens inhibit low-density lipoprotein oxidation (Zuckerman et al. 1996), decrease lipoprotein concentrations (Mosca, et al. 1998) and reduce superoxide anion production (Arnal et al. 1996).

An ex vivo experiment in isolated lung perfused by the solution without blood cells and its own original plasma favors the possibility that mechanisms other than the plasma concentration of sex hormones protect

females from increased ROS damage. The gender differences in reactivity in vitro were also shown in systemic arteries. It was explained by the difference in the handling of intracellular  $\text{Ca}^{2+}$  (Loukotova et al. 2002).

We did not find a difference in the development of pulmonary edema in the male and female experimental groups. This may indicate that gender difference does not affect pulmonary capillary permeability.

Simultaneously, it is necessary to comment that increased weight gain occurs even in control groups (Cf, Cm) which were not exposed to hot and cold ischemia. The reason why the weight of lungs increased after the initial two hours of the experiment even in the control group cannot be attributed only to the formation of pulmonary edema. Lung weight may also increase due to the increase in intravascular volume as a result of vasodilatation or vascular recruitment. Impairment of oxygen transport may result from local changes in the ventilation/perfusion ratio. (Mrazkova et al. 2015).

Our experimental measurement leads to the conclusion that female lungs are generally more resistant to ischemia-reperfusion injury after warm and cold ischemia. Therefore, it is necessary to consider, in terms of graft survival, but also from the overall economic standpoint, whether to prefer female grafts for NHBD ex vivo reperfusion lungs.

## **5. Acknowledgements**

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## **6. Conflict of interest**

There is no conflict of interest.



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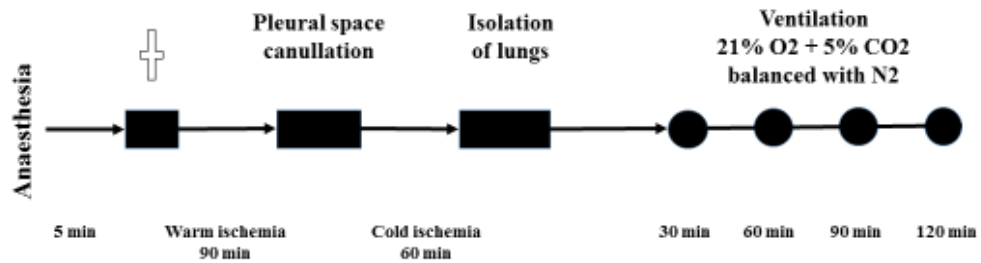
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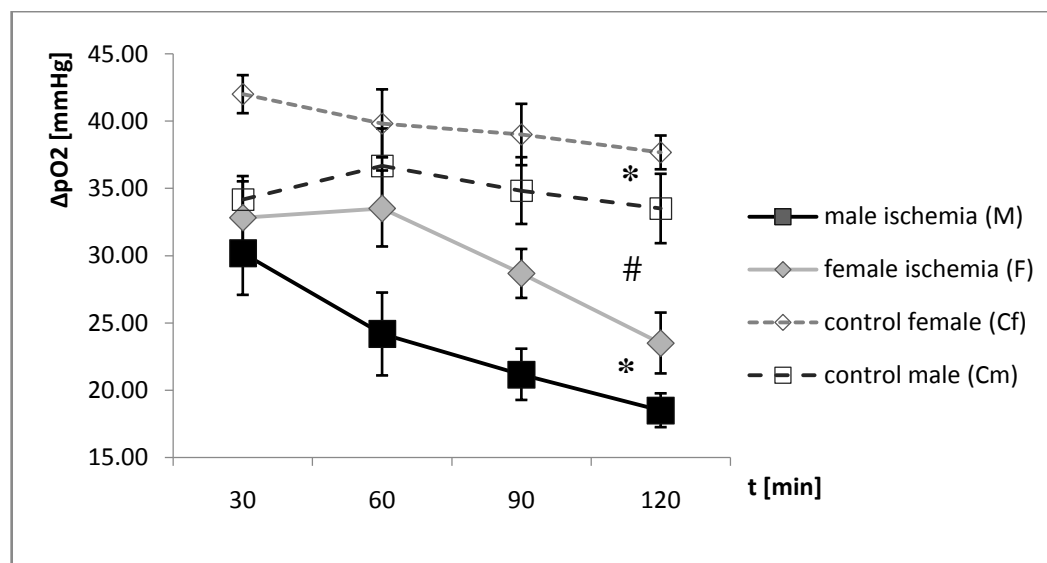
**Figure legends:**

**Fig. 1 Schematic protocol of experiment**



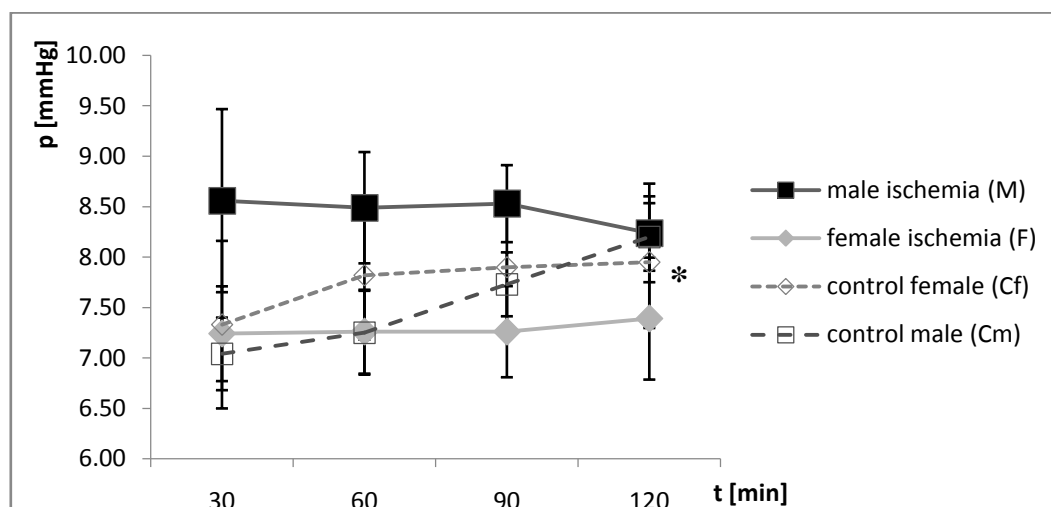
**Fig. 2 Oxygen transport ability**

We found significantly higher oxygenation capability in female rat lungs (F) compared to the male rat lungs (M) – \* $p < 0.05$ . The best oxygen transport ability, especially at the beginning of perfusion, was observed in the control female group (Cf), and not only was it significantly higher than the experimental groups (F, M) – # $p < 0.05$ , but also than the control male group (Cm) – \* $p < 0.05$ .



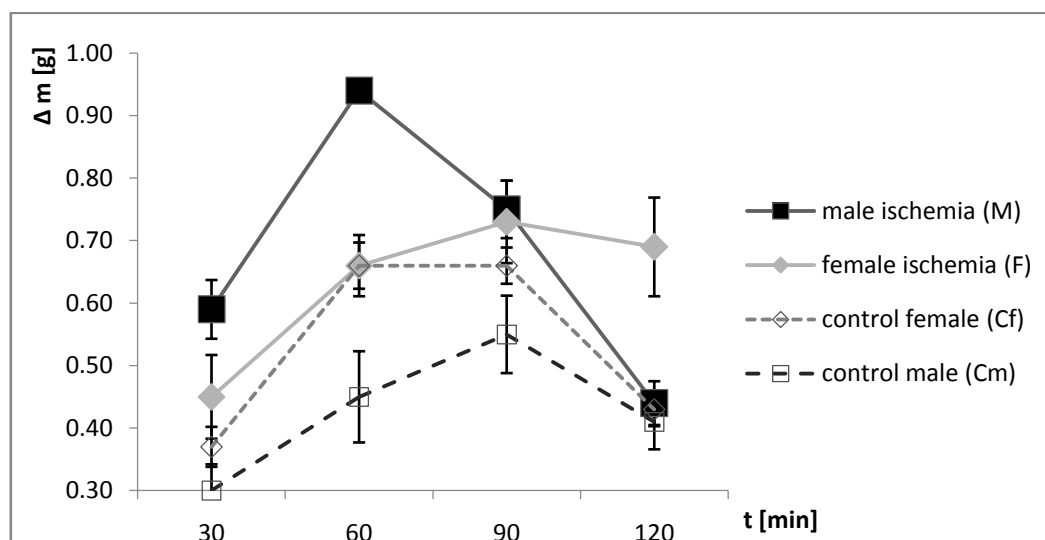
**Fig. 3 Pulmonary perfusion pressure**

Significantly lower pulmonary perfusion pressure was observed in the female experimental lungs (F) compared to the male rat group (M) (Fig. 3) – \* $p < 0.05$ . We did not notice a significant difference between the control groups (Cf, Cm) or between the experimental (F, M) and control groups (Cf, Cm).



**Fig. 4 Lung weight gain**

During observation, there was no significant difference between experimental male and female groups (F, M). The only significant difference was observed between the experimental male and control male group (M, Cm) – \* $p < 0.05$ .



**Tab. 1 Experimental and control groups – schematic protocol**

Cf – control group female rats, Cm – control group male rats, F – experimental group female rats and M – experimental group male rats

Experiment	Groups	warm ischemia 90min	cold ischemia 60min	reperfusion - 2hours experiment
		No ventilation	No ventilation	Ventilation 21% O2 + 5% CO2 balanced with N2
Control groups	Cf			x
	Cm			x
Experiment groups	F	x	x	x
	M	x	x	x

