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GENDER DIFFERENCES IN THE LONG-TERM EFFECTS OF PERINATAL HYPOXIA ON THE PULMONARY CIRCULATION IN RATS

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Short title: Late effects of perinatal hypoxia on lung vessels

Abstract

Some effects of perinatal hypoxia on the pulmonary circulation are permanent. Since pulmonary vascular sensitivity to hypoxia in adults differs between sexes, we hypothesized that gender-based variability also exists in the long-term effects of perinatal hypoxia. Rats spent 1 week before and 1 week after the birth in hypoxia (12% O_2) and then lived in normoxia. When adult, females but not males with the perinatal experience of hypoxia had right ventricle hypertrophy. To assess the role of sex hormones, some rats were gonadectomized in ether anesthesia as newborns. Compared to intact, perinatally normoxic controls, muscularization of peripheral pulmonary vessels in adulthood was augmented in perinatally hypoxic, neonatally gonadectomized males (by 85%) and much more so in females (by 533%). Pulmonary artery pressure was elevated in perinatally hypoxic, neonatally gonadectomized females (24.4 ± 1.7 mmHg) but not males (17.2 ± 0.6 mmHg). Gonadectomy in adulthood had no effect. We conclude that female pulmonary circulation is more sensitive to late effects of perinatal hypoxia, and these effects are blunted by the presence of ovaries during maturation.

Keywords:

sexual variability; ovaries; pulmonary hypertension; right ventricular hypertrophy; newborn

Introduction

Chronic hypoxia is a well-defined, clinically important cause of pulmonary hypertension. With restoration of normoxia, hypoxic pulmonary hypertension is completely reversible (16). However, if the hypoxic exposure occurs at around the time of birth, the recovery is incomplete, and subtle yet significant alterations of the pulmonary circulation persist well into the adulthood. While characterized variously in different studies, these changes can be summarized as an increased susceptibility to the development of pulmonary hypertension later in life (10, 11, 19, 20, 31, 33).

In 1997, Barer et al noticed in a preliminary report that the late effects of perinatal hypoxia appear more prominent in female than in male rats (1). In agreement with this observation, resting pulmonary artery pressure (PAP) and/or pulmonary vascular resistance in adults were slightly but significantly elevated in studies of perinatal hypoxia where pooled male and female rats were studied (10, 19) but not in experiments on males only (11).

The present study was therefore designed to test the hypothesis that the long-term effects of perinatal hypoxia on the pulmonary circulation differ between genders, and that the presence of gonads during maturation plays a prominent role in this variability. The results were reported in a preliminary form (14).

Methods

The project was reviewed and approved by the Animal Studies Committee of the Charles University Second Medical School. All procedures conformed to the European Community and NIH guidelines for using experimental animals. Wistar rats were purchased from Anlab, Prague, Czech Republic. To analyze the specific, late effects of perinatal hypoxia and gonadectomy on the pulmonary circulation, 3 experiments were performed (one main and two supplementary). Their overall design is schematically illustrated in Figure 1.

Main experiment: perinatal hypoxia and neonatal gonadectomy

In the main experiment, adult rats were divided into groups based on gender, history of perinatal hypoxia, and gonadal status. This resulted in 4 male and 4 female groups in the main experiment:

1. perinatally normoxic with intact gonads;
2. perinatally normoxic gonadectomized as newborns;
3. perinatally hypoxic with intact gonads; and
4. perinatally hypoxic gonadectomized as newborns.

In fact, the groups with the intact gonads consisted of rats that did not undergo any surgery and rats serving as sham surgery controls. As we did not find any significant differences between sham-operated and non-operated rats, their data were pooled in order to enhance clarity by reducing the large number of groups.

Perinatal hypoxia and gonadectomy

The rats were exposed to perinatal hypoxia as previously described (11, 13). Pregnant rats were placed into a normobaric hypoxic chamber (12%O_2) 1 week before the expected date of delivery (term = 3 weeks). Newborn pups were kept in the same environment together with their mothers for another 5-7 days after the birth and then kept in room air until maturity (23 weeks). Some of the pups were gonadectomized right after their removal from the hypoxic chamber in deep ether anesthesia (24). Sham surgery was performed on some of the pups under identical conditions as the gonadectomy.

Hemodynamic and morphological measurements

When the rats were adult (24 weeks old), they were anesthetized with thiopental (40 mg/kg i.p.). While they breathed room air spontaneously, their carotid artery was cannulated to measure systemic arterial blood pressure (SAP). Under oscilloscopic monitoring of pressure, the pulmonary artery was then catheterized via the right jugular vein and right ventricle as previously described (11, 15, 23) and PAP was recorded.

Mechanical ventilation with room air was then begun through a tracheostomy at ~60 breaths/min (10 cm H_2O peak inspiratory pressure, 0 cm H_2O end expiratory pressure). The chest was opened by sternotomy with extra care taken to minimize bleeding and an ultrasonic flow probe (2.5 mm SS-series with J reflector, Transonic Systems, Ithaca, NY, USA) was placed at the ascending aorta to measure aortic blood flow (T106 flowmeter, Transonic Systems) as an estimate of cardiac output (12, 23). This value

relative to body weight is reported as cardiac index (CI). The values obtained with this method are lower than cardiac output in vivo due to the anesthesia and especially the thoracotomy.

The heart was then dissected and the right and left ventricles and septum were separately weighed (8). Right ventricle to left ventricle plus septum weight ratio (RV/LV+S) was used as an index of right ventricular hypertrophy associated with pulmonary hypertension. The lungs were filled through the trachea with neutral formol solution at a pressure of 12 cm H_2O and then placed in the same solution for several days. Lung sections were then cut and stained by the hematoxylin resorcin fuchsin method. Because the muscular layer of pulmonary vessels is enclosed between two elastic laminae while the nonmuscular vessels only have one lamina, the degree of muscularization of peripheral lung vasculature was determined as previously described (16, 23) by separately counting distal pulmonary vessels (adjacent to alveoli or alveolar ducts, <300 μm in diameter) with and without double elastic lamina. All such vessels in one sagittal section of a left lung of each rat were counted (median 105 vessels). The percentage of the total that is double laminated (%DL) was then calculated (16, 23).

Supplementary experiment 1: Gonadectomy in adulthood

As the data in the main experiment showed significant effects of neonatal gonadectomy, a question arose whether for these effects to occur the gonads must be absent just at the time of the measurement in adulthood or rather during the preceding period of development. To address this issue, a supplementary experiment was

performed in which male and female rats were exposed to perinatal hypoxia identically to the rats in the main experiment, but the gonadectomy was postponed till maturity (13 weeks of age). They were then given 10 weeks to recover from the surgery before being measured.

Supplementary experiment 2: Hypoxia in adulthood

To confirm that the effects observed in the main experiment were due to hypoxia acting specifically at the perinatal period, another supplementary experiment was performed. Rats were exposed to identical hypoxia as in the main experiment (12 % O_2 for 2 weeks), but not at the perinatal period, but rather after reaching maturity (starting from 12 weeks of age). Right afterwards, some of the rats were gonadectomized. All rats then lived in room air for 10 weeks. Only RV/LV+S was determined in this supplementary experiment.

Statistical analysis

The data were analyzed using one-factor ANOVA followed by Scheffé post hoc test (StatView 5.0.1, SAS Institute, Cary, NC, USA). The Scheffé test was chosen because of its relative robustness with uneven ns between groups. Our groups varied in size because of the pooling of non-operated and sham operated controls and because not all measurements were successful in all rats (e.g. excessive bleeding before CI measurement). Actual ns in each analysis are shown in tables and figures. Statistical significance was assumed when $P < 0.05$.

Results

Late effects of perinatal hypoxia in gonad-intact males and females

While adult males were heavier than females (as expected), perinatal history of hypoxia had no effect on body weight in adulthood (Table 1). Perinatal hypoxia had no significant effect on SAP, PAP, CI, or left ventricle plus septum weight in adulthood in either sex (Table 1, Figure 2A and D). The relative weight of the right ventricle (RV/LV+S), equal in perinatally normoxic males and females, was significantly increased by the perinatal experience of hypoxia in adult females (Figure 2B). The degree of peripheral pulmonary vessel muscularization (%DL) was not significantly affected by perinatal hypoxia (Figure 2C), although in males the P value was marginal (0.058).

Perinatal hypoxia and neonatal gonadectomy

In perinatally normoxic rats, neonatal gonadectomy had no significant effect on any of the variables measured in adulthood (Table 1, Figure 2). In perinatally hypoxic males and females, neonatal gonadectomy had no significant effect on BW, SAP, CI, and left ventricle plus septum weight in adulthood (Table 1, Figure 2D). Neonatal castration had no effect on PAP and RV/LV+S in adult males with the history of perinatal hypoxia (Figure 2A and B). %DL was higher in perinatally hypoxic castrated males compared to perinatally normoxic males with intact gonads (by 85%) or gonadectomized (Figure 2C).

In females, PAP was considerably higher in perinatally hypoxic, gonadectomized rats than in all other groups (Figure 2A). Similarly, %DL was enormously elevated in adult females with the perinatal history of hypoxia and gonadectomy as compared to all other groups (by 533 % compared to perinatally normoxic intact females; Figure 2C). Neonatal ovariectomy had no further significant effect on RV/LV+S in addition to that exerted by perinatal hypoxia alone (Figure 2B).

Supplementary experiment 1: Gonadectomy in adulthood

As seen in Table 2, gonadectomy in adulthood did not have any effect in the perinatally hypoxic males or females on any of the measured variables.

Supplementary experiment 2: Chronic hypoxia in adulthood

Chronic hypoxia in adulthood did not have any long-term effect on RV/LV+S measured after recovery in normoxia in either sex regardless of the gonadal status (Table 3).

Discussion

The main new findings of the present study can be summarized in two points:

1. Hypoxia in the perinatal (but not in other) period had a permanent consequence of elevated RV/LV+S in intact females.
2. Ovariectomy at a very young age (but not in adulthood) greatly augmented the effects of perinatal hypoxia in females, so that PAP and %DL in adulthood were considerably increased. By contrast, castration of males (neonatal or adult) did not unmask any appreciable long-term effects of perinatal hypoxia on the pulmonary circulation, except an increased %DL (much less than in females).

The late effects of perinatal hypoxia on the pulmonary circulation were first reported in 1990. In a study on male rats, we found that perinatal hypoxia did not cause any significant elevation of PAP, RV/LV+S, or %DL in adulthood (in agreement with our present results), but pulmonary vasoconstrictor reactivity to acute hypoxic challenges was greatly increased by perinatal hypoxia in rats recovering from chronic hypoxia in adulthood (11). We interpreted this finding as indicating that perinatal hypoxia lead to increased susceptibility to stimuli promoting the development of pulmonary hypertension in adulthood. At the same time, Hakim and Mortola (10) reported slightly but significantly elevated vascular resistance in isolated perfused lungs of a mixed group of adult male and female rats that had been exposed to neonatal hypoxia, as compared to normoxic controls. Pulmonary vasoconstrictor reactivity to acute hypoxia was also elevated (10).

Although there are discordances in details between our earlier study (11) and that of Hakim and Mortola (10), they are both consistent with an idea that perinatal hypoxia does not cause permanent severe pulmonary hypertension, but it does increase the susceptibility of adult lung vasculature to insults later in life. This concept was subsequently confirmed several times in various models (2, 17, 19, 20, 33), including a study in humans (31). Thus, our present finding of definite, yet relatively weak, effects of perinatal hypoxia on baseline characteristics of the pulmonary circulation in intact adult rats is consistent with these previous reports.

In the present experiment, the animals with the history of perinatal hypoxia were studied when much older (6 months) than rats in the previous reports (1.5-4 months) (2, 10, 11, 17, 19, 20). The fact that the effects of perinatal hypoxia on the pulmonary circulation were still evident after a normoxic interval this long suggests that they may be permanent rather than only slowly reversible.

Chronic hypoxic pulmonary hypertension in adulthood is less severe in females than in males (27). This might be, in part, related to less severe acute hypoxic pulmonary vasoconstriction in females (5, 34). It is therefore somewhat surprising in our present study that with perinatal hypoxia, the late effects are slightly more prominent in intact females than males. As the greater resistance of female pulmonary circulation to hypoxia in adulthood is caused mostly by female sex hormones (5, 34), our logical next step was to test the role of gonads in the gender variability of the late effects of perinatal hypoxia. We found that early ovariectomy did not eliminate the gender

differences - as is the case with hypoxia in adulthood (34) - but, on the contrary, exacerbated them. These data imply two interesting phenomena. One is that ovarial function is protective not only against hypoxic pulmonary hypertension in adulthood, but also against the late effects of perinatal hypoxia. The second implication is that without the protective action of the ovaries, pulmonary vasculature of female rats is much more susceptible to adverse, long-term effects of perinatal hypoxia. Thus, there are marked gender differences in the postnatal development of the pulmonary circulation that have both gonads-dependent and -independent components.

The protective effects of female sex hormones, especially estrogens, on the circulation in general (6, 22, 35) and on the pulmonary vessels in particular (25, 26, 28) have been studied quite extensively. By affecting numerous signaling systems, such as nitric oxide, prostacyclin, endothelin, angiotensin II and various growth factors, estrogens decrease vascular smooth muscle tone and inhibit vascular wall growth (3, 4, 6, 7, 18, 21, 32). It is likely that one or more of these mechanisms may also be involved in the protective effect of the ovaries against the long-term effects of perinatal hypoxia on the pulmonary circulation. However, despite the attractiveness of estrogens as the agents of protection in this situation, our study does not positively prove their role. Possible involvement of other ovarial hormones cannot be excluded. Hypothetically, the changes in gonadotropin hormones secondary to gonadectomy might also play a role. Androgen involvement seems unlikely because their levels in females are markedly lower than in males, where their removal had much less effect.

Because estrogens relax vascular wall and inhibit its growth (6, 22, 35), one likely explanation for the protective effect of the ovaries could have been that they mask the effects of perinatal hypoxia by continually stimulating the synthesis of vasodilators in adulthood, including the time of our measurements. Our data indicate that this is not the case – for the protective effect to occur, the ovaries must be functional during maturation, but not at the time of measurement in adulthood. Thus, the ovaries appear to act on the pulmonary vasculature during development to reduce the long-term hypertensive consequences of perinatal hypoxia.

Although the influence of neonatal gonadectomy on the late effects of perinatal hypoxia were much more prominent in female than in male rats, they were not completely absent in males. Namely, the muscularization of peripheral pulmonary vessels was augmented in adult males with the history of perinatal hypoxia if they had been gonadectomized as pups, albeit much less so than in females. If indeed the protective effect of the ovaries in females is mediated by estrogens, as speculated above, then the same mechanism might also be in action in males. The much smaller protective effect of the testes as compared to the ovaries would correspond to their much smaller estrogen production. Alternatively, an independent, yet smaller protective effect of testosterone cannot be excluded based on our present data.

In adults, the properties of pulmonary (and other) vessels themselves are similar between males and females and the advantage of a lower susceptibility to hypertension is imparted on the female vasculature by the female sex hormones. The

most unusual finding of the present study is therefore that without the protective effect of the ovaries, the female pulmonary vessels are not equal to the male ones in their sensitivity to the late effect of perinatal hypoxia (females are affected much more). The cause of this gender difference is unknown, but it appears that it is an intrinsic property of the affected cells of the vascular wall that is not governed by sex hormones.

The gender differences in the late effects of perinatal hypoxia and gonadectomy are selective to pulmonary circulation: SAP did not differ among the groups regardless of gender, gonadal status, or perinatal history (Tables 1 and 2). This is in agreement with a recent study showing equal SAP in perinatally normoxic and hypoxic rats (29). It is possible that the pulmonary selectivity is due to the substantial remodeling of the pulmonary vasculature that occurs at the time of birth and to the fact that chronic hypoxia causes pulmonary, but not systemic, hypertension. However, the mechanism of acute hypoxic sensing appears to be permanently altered by perinatal hypoxia both in the pulmonary (vasoconstriction) (10, 11) and systemic (vasodilation) (29) vascular beds. Our data do not exclude the possibility that stimuli more relevant to the systemic circulation, acting at the perinatal period, may have late effects similar to those observed in this study with hypoxia and the pulmonary circulation.

The prominent vulnerability of female pulmonary circulation to the late consequences of perinatal hypoxia and the protective role of functional ovaries resonate with a clinical experience that the much feared primary pulmonary hypertension is about twice as frequent in women than in man and that it is typically a disease of reproductive age (9,

30). While there is currently no data to support a mechanistic connection between these observations, it is tempting to speculate that a history of insults during a critical period of development, such as perinatal hypoxia, might combine with female sex hormone alterations to increase the susceptibility to primary pulmonary hypertension. Further examination of this speculation appears warranted.

In conclusion, we found that perinatal hypoxia causes long-term alterations of the pulmonary circulation and right heart that are more pronounced in female than in male rats. Chronic hypoxia in adulthood does not have such delayed effects. Gonadectomy at a very young age, but not in adulthood, greatly augments the late pulmonary hypertensive effects of perinatal hypoxia, but only in females. Thus, male and female pulmonary vessels inherently differ in sensitivity to perinatal hypoxia. During maturation, ovaries exert a protective influence against the permanent consequences of perinatal hypoxia in the lung vessels.

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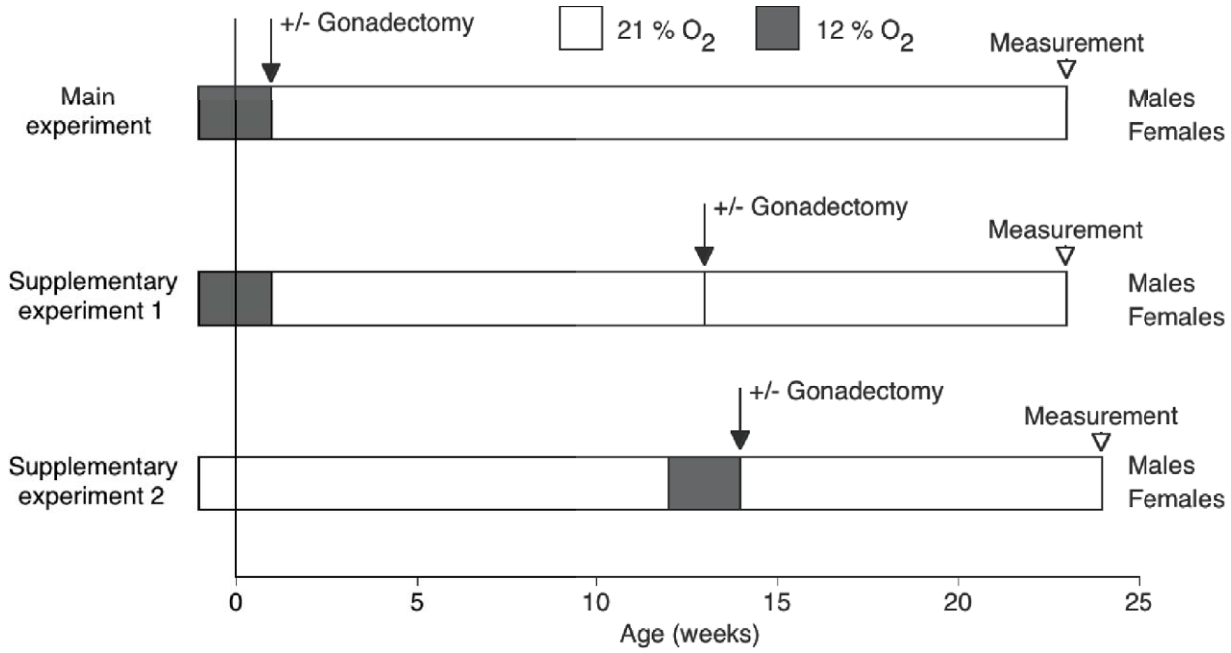

Figure 1: Experimental groups in the main and 2 supplementary experiments.

In each experiment, males and females were studied separately. In the main experiment, the rats were exposed to hypoxia (dark bar) for 1 week before and 1 week after the birth, then grew up in normoxia (white bar) until studied at 23 weeks of age (Measurement). Some, but not all, of them were gonadectomized at the end of the hypoxic exposure (+/- Gonadectomy). In supplementary experiment 1, the male and female rats were exposed to the same environments as the rats in the main experiments, but the gonadectomy of some of them was postponed until 13 weeks of age. In supplementary experiment 2, male and female rats were born and matured in normoxia and were exposed to hypoxia for 2 weeks when adult. Some of them were then gonadectomized and all were given 10 weeks in normoxia to recover.

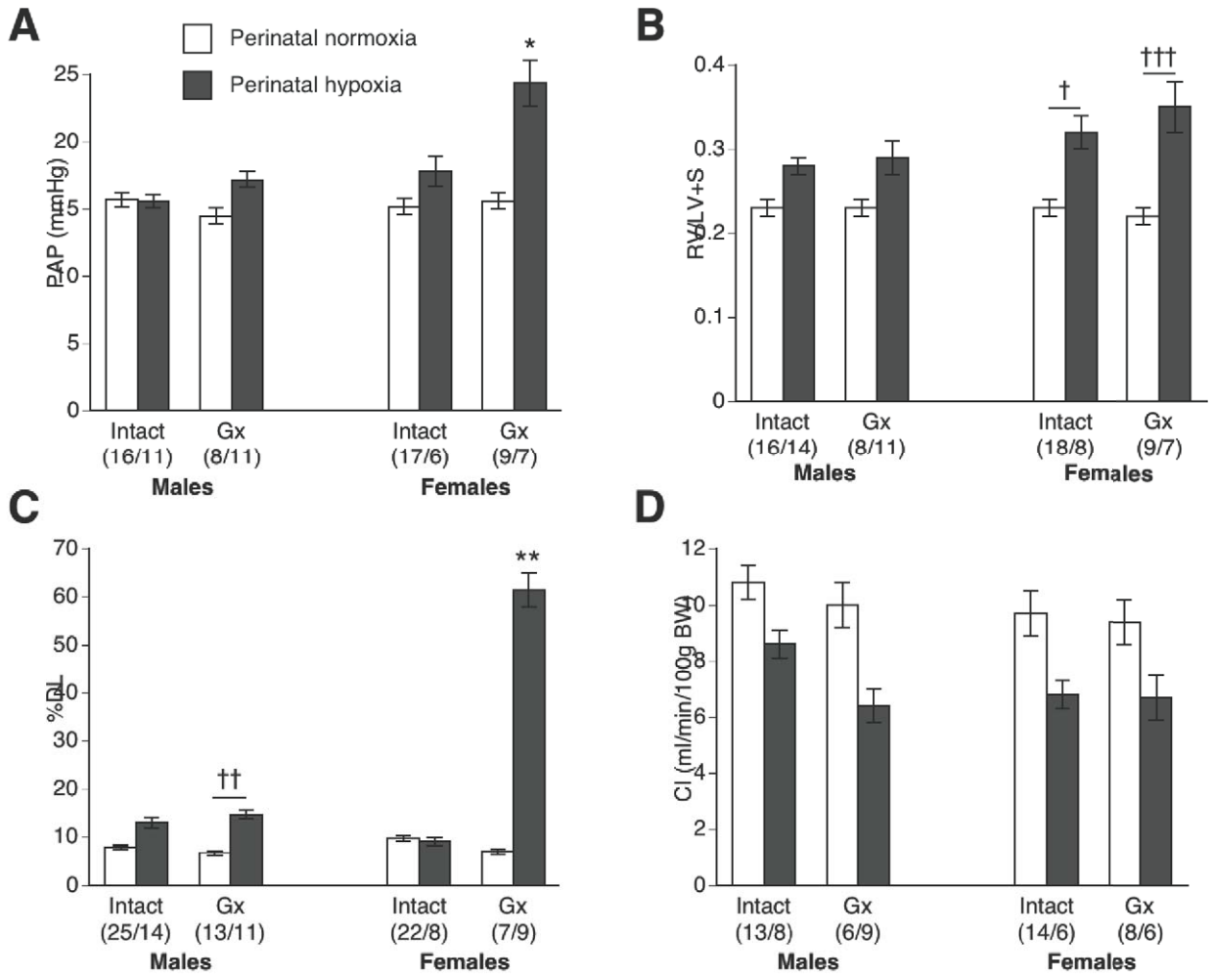

Figure 2: Effects of perinatal hypoxia and neonatal gonadectomy on the pulmonary circulation, cardiac index and right ventricle in adulthood.

Intact, rats with intact gonads; Gx, gonadectomized rats; white columns, animals born and raised in normoxia; dark columns, perinatally hypoxic rats. In parentheses is n of perinatally normoxic / n of perinatally hypoxic animals. Data are means ± SEM.

(**A**) Mean pulmonary arterial blood pressure (PAP) is not significantly affected by perinatal hypoxia in intact males and females, but is elevated in perinatally hypoxic females that had been ovariectomized as newborns.

(**B**) The relative weight of the right ventricle (right ventricle to left ventricle plus septum wet weight ratio, RV/LV+S) is increased by perinatal hypoxia in females but not in males. Left ventricle plus septum weights were unaffected by perinatal hypoxia or neonatal gonadectomy (Table 1).

(**C**) Muscularization of peripheral pulmonary vessels, expressed as percentage of double laminated (i.e. muscularized) peripheral vessels (%DL) is markedly increased in perinatally hypoxic females that had been ovariectomized as newborns. A similar change in males is much less prominent.

(**D**) The tendency for lower cardiac index estimate (CI) in perinatally hypoxic as compared to perinatally normoxic rats did not reach statistical significance.

* $P<0.005$ differs from all other groups

** $P<0.0001$ differs from all other groups

† $P<0.01$

†† $P<0.005$

††† $P<0.0001$

Table 1: Body weight (BW), mean systemic arterial blood pressure (SAP), and left ventricle plus septum wet weight (LV+S) in intact and neonatally gonadectomized rats.

Gonads	**Sex**	**Perinatal O_2**	**BW** (g)	**SAP** (mmHg)	**LV+S** (mg)
Intact	Males	Normoxia	505±11 (16) *	114±3 (16)	920±22 (16) *
		Hypoxia	507±15 (14) *	119±3 (12)	903±31 (14) *
	Females	Normoxia	311±12 (18)	114±2 (17)	835±41 (18)
		Hypoxia	345±33 (8)	123±4 (7)	783±26 (8)
Gonadectomy (neonatal)	Males	Normoxia	477±17 (8) **	117±4 (8)	612±16 (8) **
		Hypoxia	466±15 (11)	110±4 (11)	600±47 (11)
	Females	Normoxia	356±32 (9)	109±5 (9)	640±21 (9)
		Hypoxia	391±17 (7)	111±4 (7)	647±29 (7)

Data are means ± SEM. In parentheses are numbers of rats.

* $P<0.001$ males differ from corresponding female group

** $P<0.05$ males differ from corresponding female group

Table 2: Gonadectomy in adulthood does not affect systemic and pulmonary circulation of perinatally hypoxic male and female rats.

Gonads	Sex	BW (g)	SAP (mmHg)	PAP (mmHg)	CI (ml/min/ 100 g BW)	RV/LV+S
Intact	Males	603±12* (18)	127±2 (18)	15.6±0.7 (16)	7.8±0.4 (15)	0.27±0.01 (18)
	Females	364±19 (18)	123±3 (17)	15.9±0.4 (15)	6.7±0.6 (12)	0.27±0.01 (18)
Gonadectomy (in adulthood)	Males	563±16* (10)	126±3 (10)	14.9±0.8 (7)	7.2±0.4 (6)	0.26±0.01 (10)
	Females	392±14 (9)	125±3 (9)	15.7±0.8 (7)	6.0±0.6 (7)	0.27±0.02 (9)

All groups in this table were perinatally hypoxic and measured when adult. BW, body weight; SAP, mean systemic arterial blood pressure; PAP, mean pulmonary arterial blood pressure; CI, open-chest estimate of cardiac index; RV/LV+S, right ventricle to left ventricle plus septum wet weight ratio. Data are means ± SEM, in parentheses are numbers of rats.

* $P<0.001$ males differ from corresponding female group

Table 3: Chronic hypoxia in adulthood does not have any delayed effect on relative right ventricle weight.

Gonads	**Sex**	**Hypoxia in adulthood**	**BW** (g)	**RV/LV+S**
Intact	Males	No	653±11 (7)*	0.29±0.02 (7)
		Yes	593±17 (8) *	0.30±0.02 (8)
	Females	No	378±19 (4)	0.29±0.01 (4)
		Yes	363±5 (7)	0.29±0.02 (7)
Gonadectomy (in adulthood)	Males	Yes	586±16 (8) *	0.30±0.01 (8)
	Females	Yes	402±11 (7)	0.32±0.03 (7)

All rats in this table were measured 10 weeks after a 2-week exposure to hypoxia in adulthood. BW, body weight; RV/LV+S, right ventricle to left ventricle plus septum wet weight ratio. Data are means ± SEM, in parentheses are numbers of rats.

* $P<0.0001$ males differ from corresponding female group