Restriction of placental function alters heart development in the sheep fetus

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**Running Head:** Heart development in the growth restricted fetus

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ABSTRACT

Placental insufficiency, resulting in restriction of fetal substrate supply, is a major cause of intrauterine growth restriction (IUGR) and increased neonatal morbidity. Fetal adaptations to placental restriction maintain the growth of key organs including the heart, but the impact of these adaptations on individual cardiomyocytes is unknown. Placental and hence fetal growth restriction was induced in fetal sheep by removing the majority of caruncles in the ewe before mating (placental restriction, PR). Vascular surgery was performed on 13 Control and 11 PR fetuses at 110-125d gestation (term=150±3d). PR fetuses with a mean gestational PO₂<17mmHg were defined as hypoxic. At post mortem (<135 or >135d), fetal hearts were collected, and cardiomyocytes isolated and fixed. Proliferating cardiomyocytes were counted by immunohistochemistry of Ki67 protein. Cardiomyocytes were stained with methylene blue to visualise the nuclei and the proportion of mononucleated cells, length and width of cardiomyocytes were measured. PR resulted in chronic fetal hypoxia, IUGR and elevated plasma cortisol concentrations. Although there was no difference in relative heart weights between Control and PR fetuses, there was an increase in the proportion of mononucleated cardiomyocytes in PR fetuses. Whilst mononucleated and binucleated cardiomyocytes were smaller, the relative size of cardiomyocytes when expressed relative to heart weight was larger in PR compared to Control fetuses. The increase in the relative proportion of mononucleated cardiomyocytes and the relative sparing of the growth of individual cardiomyocytes in the growth restricted fetus are adaptations which may have long term consequences for heart development in postnatal life.

Keywords: Fetus, hypoxia, cardiomyocyte, fetal growth restriction, hyperplasia, hypertrophy, placental restriction
INTRODUCTION

Low birth weight is associated with an increased risk of heart disease in adult life (5); however, few studies have investigated the development of cardiomyocytes in the hearts of low birth weight fetuses. Fetal heart development involves proliferation and growth of cardiomyocytes. Heart growth early in development occurs via hyperplastic growth of mononucleated cardiomyocytes (45). There is a transition from hyperplastic to hypertrophic growth as increasing numbers of cardiomyocytes become binucleated and are terminally differentiated by karyokinesis in the absence of cytokinesis (9, 22). In the human heart, most cardiomyocytes undergo binucleation during fetal life, with 90% of cardiomyocytes being binucleated late in gestation and up to 97% being binucleated by 7wks after birth (1). Thus at birth, the human heart contains almost the full complement of cardiomyocytes that it will have for life (50) and, this is important as there is a limited capacity for cellular regeneration within the postnatal heart after injury (34, 44). The sheep is similar to the human in that binucleation of cardiomyocytes also occurs predominantly before birth, with hyperplastic growth of the cardiomyocytes occurring before 110d gestation (term, 150±3d), followed by a transition to hypertrophic growth by term (7, 43). Several studies have investigated the signals for cardiomyocyte development and found that IGF-1 (46), angiotensin (47) and cortisol (17) each can stimulate hyperplastic growth and that phenylephrine (46) and cortisol (28) can also cause hypertrophic growth in fetal sheep cardiomyocytes.

Given the relative maturity and limited ability for regeneration of the heart at birth (34, 50), environmental factors that alter the timing of cardiomyocyte binucleation in late gestation are likely to have long lasting consequences for heart growth and function (27). An example of an important change in the fetal environment which may impact on cardiomyocyte development is intrauterine growth restriction (IUGR). In humans, IUGR is defined as birth weight below the tenth centile (8) and is associated with chronic hypoxia (12)
and increased plasma cortisol concentrations (13) as well as increased perinatal mortality (16) and neonatal morbidity (15, 18). Despite a degree of cardiac sparing, IUGR is also associated with altered structural development of the heart (19) and heart disease (5, 30) in postnatal life.

In one experimental model of IUGR, exposure to chronic hypoxia (10% inspired air) during late gestation in pregnant rats, resulted in an increase in relative heart weight (3) and a decrease in DNA content in the heart at birth (33). Importantly, the IUGR offspring also had an increased vulnerability to ischemia-reperfusion injury (25) in adult life. There was also an increase in the proportion and in the size of binucleated cardiomyocytes at birth in this model (3). Given the significant differences among species in the developmental trajectory of cardiomyocyte development relative to birth, it is important to determine the impact of IUGR on fetal heart development in a species, like the human, where cardiomyocyte binucleation occurs before birth. In the sheep, surgical ablation of the majority of uterine caruncles before conception results in a reduction in placental mass and fetal growth throughout gestation (2), offering an excellent model of fetal growth restriction. Placentally restricted (PR) sheep fetuses have a similar arterial blood gas and hormonal profile (hypoxemia, hypercapnia, hypoglycaemia, increased plasma cortisol concentrations (29, 38)) compared to growth restricted human fetuses (13).

In the present study, we aimed to determine the impact of PR on the development of cardiomyocytes in the fetal sheep during late gestation. Cortisol is elevated in the PR fetus (37) and also stimulates hyperplastic and hypertrophic growth of cardiomyocytes (17, 28, 39). As there is a prepartum rise in cortisol in both the human (49) and sheep (32, 37), we collected hearts from fetal sheep at two stages in late gestation, i.e. before (<135d) and after (>135d), the prepartum increase in fetal cortisol (35). We hypothesised that PR, possibly acting via an increase in circulating cortisol concentrations, would result in a change in the
proportion and size of binucleated cardiomyocytes present in the heart of the growth restricted fetal sheep in late gestation.
MATERIALS AND METHODS

All experiments were performed according to guidelines of both the University of Adelaide and the University of South Australia Animal Ethics Committees.

Animals and Surgery

The majority of uterine caruncles were removed in 11 Merino ewes prior to conception as previously described (2, 11, 14). The ewes’ recovery from surgery was observed for 4-7d. After 10wk, these 11 plus 18 Control ewes entered a mating program. At 110-125d, surgery was performed in 13 Control and 11 PR fetuses under aseptic conditions with general anaesthesia induced by sodium thiopentone (1.25g; Pentothal, Rhone Merieux, Pinkenba, Qld, Aus) and maintained by inhalation of halothane (2.5-4%) in oxygen. Briefly, vascular catheters (Critchley Electrical Products, Silverwater, Aus) were inserted as previously described in the maternal jugular vein, the fetal femoral and carotid arteries, jugular vein, and the amniotic cavity (14, 31). Fetal catheters were exteriorised through a small incision in the ewe’s flank. At surgery, antibiotics were administered to the ewe (153.5mg Procaine penicillin, 393mg benzathine penicillin; 500mg dihydrostreptomycin, Lyppards, Adelaide, Aus) and fetus (150mg Procaine penicillin, 112.5mg benzathine penicillin; 250mg dihydrostreptomycin, Lyppards, Adelaide, Aus). Antibiotics were administered intramuscularly to each ewe for 3d after surgery and to each fetus intraamniotically (500mg ampicillin, Lyppards, Adelaide, Aus) for 4d after surgery. Animals were allowed to recover from surgery for at least 4d prior to experimentation.
Arterial Blood Gas Measurements

Fetal carotid arterial blood gas samples (0.5ml) were collected daily for the measurement of PO$_2$, PCO$_2$, pH, oxygen saturation (SO$_2$) and hemoglobin (Hb) at 39°C with an ABL 520 analyser (Radiometer, Copenhagen, Denmark) calibrated for sheep blood.

Post Mortem

At the time of mating, ewes were randomly allocated for post mortem at <135d or >135d. Therefore, post mortem examination was performed at 132-134 or 137-141d gestation. Ewes were killed with an overdose of sodium pentobarbitone (8g; Vibrac Australia, Peakhurst, Aus). The uterus was removed by hysterectomy and the fetus removed. The umbilical cord was cut and the fetus was weighed. The heart was quickly dissected and weighed. Heparin (20,000IU in 10ml saline) followed by saturated KCl (10ml) were perfused through the coronary vessels via the aorta. The mean time from maternal death to perfusion of the fetal heart with KCl was 11.2±0.6min.

Cardiomyocyte Dissociation

Fetal hearts were suspended in a Langendorf apparatus and perfused through the aorta with warmed (37°C), oxygenated Tyrode’s buffer (4) with a heating pump (Paratherm II, Julabo, Schwarzwald, West Germany) for 11.4±0.4min at a rate of 10.6ml/min using a Minipulse 3 pump (Gilson, Villeirs, France) to clear blood from the coronary vessels. Collagenase (120units/ml, Lot #X3M6745, Worthington Biochemical Corporation, Lakewood, NJ, USA) and protease (10mg/200ml; Lot #083K0799, Sigma, Castle Hill, NSW, Aus) solution was perfused for 14.4±1.5min at 25.8±0.6°C to digest the extracellular matrix as previously described (17, 20, 21, 46, 47). Approximately 300ml of Kraftbrühe (KB) buffer (21, 47) was then perfused through the heart to flush out these enzymes. The entire right and
left ventricle free walls and the septum were dissected and placed in Falcon tubes with KB buffer, triturated and cardiomyocytes collected. Cardiomyocytes were fixed in a 1% paraformaldehyde solution for storage.

**Cardiomyocyte Characterization**

**Ratio of Mononucleated to Binucleated Cardiomyocytes:** Nuclei of fixed cardiomyocytes were stained with methylene blue. The percentage of mononucleated cardiomyocytes was determined in each ventricle from each fetus by counting the number of mononucleated cardiomyocytes in a total of 200 cardiomyocytes (6, 7) using an Olympus VANOX-T microscope (Olympus Optical Co. Ltd, Tokyo, Japan). Counters were blinded and inter- and intra-observer variability were <10%.

**Hyperplastic Cardiomyocyte Growth:** To determine the proportion of proliferating cells in each ventricle (17, 40), immunohistochemistry, using a Ki67 antibody (1:100; mouse anti-human monoclonal antibody; DAKO, Dianova, Germany), was performed on fixed cells. Ki67 is a cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G₁, S, G₂ and M phase) but is absent in resting cells (G₀) (23, 24, 41). Cardiomyocytes were treated with 3% hydrogen peroxide for 20min to remove endogenous peroxidase activity. A Histostain-Plus Broad Spectrum kit (85-9043, Lot # 30276167, Zymed Laboratories Inc, San Francisco, USA) was utilised with DAB for visualisation of positively stained nuclei. Nuclei were counterstained with 1µl of 1% methylene blue and 10µl of cell suspension was placed on a slide for analysis. Random, non-repeating fields were analysed to determine the percentage of cardiomyocytes undergoing proliferation from the number of mononucleated cardiomyocytes that were positive for Ki67 out of 200 mononucleated cardiomyocytes using an Olympus VANOX-T microscope (Olympus Optical Co. Ltd, Tokyo, Japan). Counters were blinded and inter- and intra-observer variability were <10%.
Hypertrophic Cardiomyocyte Growth: 10µl of fixed cardiomyocytes were stained with 1µl of 1% methylene blue on a slide, visualised with an Olympus VANOX-T microscope (Olympus Optical Co. Ltd, Tokyo, Japan) and images captured with an Olympus C-35AD-4 camera (Olympus Optical Co. Ltd, Tokyo, Japan). From these images, length and width measurements (4, 17, 46, 47) were made of 50 mononucleated and 50 binucleated cardiomyocytes (6) using AnalySIS® software (Soft Imaging System, Adelaide, Aus) calibrated using a graticule to 2µm and a mean determined for each animal. The length of the cardiomyocyte was the longest distance from one tip of the cardiomyocyte to the other and the width was across the midline of the nucleus in mononucleated cardiomyocytes and midway between the two nuclei in binucleated cardiomyocytes. Inter- and intra-observer variability were less than 5%.

Cortisol Radioimmunoassay: Total plasma cortisol concentration was measured in extracts, using an 125I radioimmunoassay kit (GE Healthcare, Sydney, Australia) as previously described (48). The average efficiency of recovery of 125I cortisol using dichloromethane extraction was 90%. The sensitivity of the assay was 0.39nmol/l. The rabbit anti-cortisol antibody cross-reacted <1% with cortisone and 17-hydroxyprogesterone and <0.01% with aldosterone, pregnenolone, estradiol and progesterone. The inter- and intra- assay coefficients of variation were less than 10%.

Statistical Analyses

All data are presented as the mean ± SEM. A probability value of 5% (P<0.05) was considered significant.
**Experimental Groups:** Animals were divided into treatment (Control vs. PR) and age (<135d vs. >135d) groups (Table 1). All Control fetuses had a mean gestational arterial PO$_2$ of greater than 17mmHg or, if PO$_2$ measurements were not available (<135d, n=2; >135d, n=3), their body weight at post mortem was within two standard deviations of the mean of the body weight of all Control fetuses in the relevant age group (29). Statistical analysis was performed to validate this approach and there was no significant difference (unpaired t test) in fetal weight, heart weight, proportion of mononucleated cardiomyocytes, percent Ki67+ mononucleated cardiomyocytes or size of cardiomyocytes between the fetuses that were included on the basis of mean gestational PO$_2$ or body weight at birth. All PR fetuses had a mean gestational PO$_2$ below 17mmHg and were therefore defined as chronically hypoxic (11, 14) and, in addition, were growth restricted. Table 1 shows fetal number and sex in each group. A recent study shows no difference in cardiomyocyte parameters between twins and singletons (21).

**Blood Gas and Fetal Plasma Cortisol Concentration Data:** Mean gestational arterial PO$_2$, O$_2$ saturation (SO$_2$), hemoglobin (Hb), PCO$_2$ and pH were calculated as the mean of the values for all samples collected between surgery and post mortem. A two way Analysis of Variance (ANOVA) was used to determine differences in the mean fetal blood gases, pH, arterial oxygen content (Oxygen Content =(PO$_2$*0.003)+[Hb]*(SO$_2$/100)*1.39 ml/dl) (14)) and plasma cortisol concentration between treatment (Control vs. PR fetuses) and age (<135d vs. >135d) groups.

**Cardiomyocyte Measurements:** A multifactorial ANOVA was used to determine differences in the percentage of mononucleated cardiomyocytes, percentage of Ki67 positive mononucleated cardiomyocytes and length and width of both mononucleated and binucleated cardiomyocytes with treatment (Control vs. PR); age (<135d vs. >135d) and ventricle (left vs.
right) as the specified factors (SPSS, Chicago, USA). Linear regression was performed on cardiomyocyte data to investigate relationships with mean gestational PO\(_2\) using SigmaPlot (SPSS, Chicago, USA).
RESULTS

Effects of PR on fetal growth, gender and number of fetuses

The gestational age at post mortem was not different between Control and PR fetuses in the <135d or the >135d groups (Table 2). PR fetuses were smaller than Control fetuses at both <135d and >135d (P<0.01). All PR fetuses weighed less than the 10th centile of Control fetuses in each age group (<135d, <3.15kg; >135d, <4.25kg). Whilst Control fetuses were heavier after 135d than before 135d, this was not the case for PR fetuses. Heart weight was lower in PR than Control fetuses at both <135d and >135d (P<0.01), however there was no difference in the relative heart weight between treatment groups at either gestational age range. There was a significant difference in the number of fetuses per pregnancy between the Control and PR groups at both ages (P<0.01; Table 1). There was no difference in the gender distribution between the Control and PR groups or across gestational age.

Effects of PR on fetal arterial blood gases

Mean gestational PO₂ (P<0.01), SO₂ (P<0.01) and O₂ content (P<0.01), were lower in PR than Control fetuses at both <135d and >135d (Table 3). Oxygen content increased with gestational age in the Control fetuses and decreased with gestational age in the PR fetuses (P<0.05).

Plasma cortisol concentrations

Fetal plasma cortisol concentration increased with gestational age in Control fetuses (<135d, 5.5±1.6nmol/l; >135d, 12.9±3.1nmol/l; P<0.05) and was higher in PR fetuses (<135d, 18.5±2.6nmol/l; >135d, 17.3±4.1nmol/l) compared to Control fetuses (P<0.01). There was no relationship between plasma cortisol concentration and mean gestational PO₂,
the percent mononucleated cardiomyocytes, Ki67+ cardiomyocytes or the size of either mononucleated or binucleated cardiomyocytes.

**Effects of PR on the proportion and proliferation of mononucleated cardiomyocytes**

The proportion of mononucleated cardiomyocytes was higher in PR than Control fetuses ($P<0.01$; Figure 1A). There was no effect of gestational age on the percentage of mononucleated cardiomyocytes present in either ventricle and there was no difference in the percentage of mononucleated cells between the right and left ventricle. There was an inverse direct correlation between mean gestational PO2 and the percentage of mononucleated cardiomyocytes in the right ($P<0.01$, $r^2=0.272$) and the left ventricle ($P<0.01$, $r^2=0.475$; Figure 1B).

The percentage of mononucleated cardiomyocytes which expressed Ki67 was similar in Control and PR fetuses (Figure 2B). There was no effect of age on the percentage of Ki67 positive mononucleated cardiomyocytes and there was also no difference between the right and left ventricles in the proportion of mononucleated cardiomyocytes which expressed Ki67. There was no relationship between mean gestational PO2 and percent Ki67+ mononucleated cardiomyocytes.

**Effects of PR on the morphometry of cardiomyocytes**

Mononucleated cardiomyocytes from PR fetuses were significantly shorter ($P<0.05$) and narrower ($P<0.05$) than Control fetuses. The mononucleated cardiomyocytes were also shorter ($P<0.01$) and narrower ($P<0.01$) in the left than in the right ventricle in both age groups (Table 4).
Binucleated cardiomyocytes were significantly narrower in hearts from PR than Control fetuses ($P<0.05$; Table 4) and became more narrow with increasing gestational age ($P<0.01$). In contrast, there was no difference in the length of the binucleated cardiomyocytes in the PR and Control fetuses. In both groups, binucleated cardiomyocytes were longer ($P<0.01$) and wider ($P<0.05$) in the right compared to the left ventricle.

When the size of the cardiomyocytes was expressed relative to heart weight, both mononucleated and binucleated cardiomyocytes were wider (mononucleated, $P<0.01$, Figure 3A; binucleated, $P<0.01$, Figure 3C) and longer (mononucleated, $P<0.01$, Figure 3B; binucleated, $P<0.01$, Figure 3D) relative to heart weight in the PR group compared to Control fetuses. In addition, with increasing gestational age, there was a decline in the relative width of both mononucleated ($P<0.05$) and binucleated ($P<0.05$) cardiomyocytes in both treatment groups. There was no relationship between fetal plasma cortisol concentration and the relative cardiomyocyte size. There was, however, a relationship between mean gestational PO$_2$ and the dimensions of the cardiomyocytes relative to heart weight in both the right and left ventricle ($P<0.05$; Figure 3E and 3F).
DISCUSSION

This study investigated the impact of placental and fetal growth restriction on cardiomyocyte development. As expected, the PR fetuses were growth restricted, hypoxic and relatively hypercortisolemic compared with age matched Control fetuses (11, 14, 37, 42). Importantly, we have found that whilst the relative heart weight was not different in the PR compared to the Control fetuses there was an increased proportion of mononucleated cardiomyocytes present in the hearts of PR fetuses both before and after 135d gestation. This increase occurred in the absence of a change in the relative proportion of proliferating mononucleated cardiomyocytes. Whilst mononucleated and binucleated cardiomyocytes were smaller in the PR fetuses, the relative size of these cardiomyocytes when expressed relative to heart weight was larger in the PR compared to the Control fetuses. These novel findings highlight that chronic fetal substrate restriction has a significant impact on the development of the heart at the cellular level in a large animal model in which the development of the cardiomyocyte profile is virtually complete by birth, as in the human.

In the present study, we found that ~40% of cardiomyocytes were mononucleated at both gestational age ranges in the Control fetuses. This is consistent with previous studies which have reported that there are ~30-50% mononucleated cardiomyocytes present in the fetal sheep heart at this stage of gestation (4, 7, 17, 46). We found that there was no change in the proportion of mononucleated cardiomyocytes present in either the PR or Control fetuses across the gestational age range of animals used in this study. This is not inconsistent with previous studies in which the range of values cited for the proportion of mononucleated cardiomyocytes present during late gestation span 30-75% at 133-4 d gestation and between 20-40% after 140d gestation (7). The major finding of the present study is the increased proportion of mononucleated cardiomyocytes present in both ventricles of hearts from the PR fetus, compared to Control fetuses. One interpretation of this finding is that there has been a
delay in binucleation of the cardiomyocytes in the growth restricted fetus. Interestingly recent studies have reported that there was also a lower incidence of cardiomyocyte proliferation (26) and binucleation in the fetal heart after exposure to uteroplacental embolisation between 110 and 130d gestation in the fetal sheep (6, 26). Placental embolisation results in acute changes in fetal arterial PO2 from the onset of embolisation, and results in a 30% decrease in fetal growth by 130d gestation. In our study, we found that there was a direct relationship between the degree of fetal hypoxaemia and the proportion of mononucleated cardiomyocytes present in the fetal heart. There was no difference, however, in the expression of a marker of cellular proliferation within the mononucleated cardiomyocytes between the PR and Control fetuses. Thus it appears that fetal hypoxaemia, experienced either as a result of acute placental failure in late gestation (as in placental embolisation) or as a restriction of placental growth and function from conception (as in the PR fetuses) results in a delay in cardiomyocyte binucleation. Acute placental restriction, however, results in a decrease in cellular proliferation within the fetal heart, in contrast to chronic placental insufficiency which does not result in changes in cardiomyocyte proliferation.

Interestingly, both mononucleated and binucleated cardiomyocytes were smaller in PR compared to Control fetuses. Although cardiomyocytes were smaller in the heart of the PR fetuses, when cardiomyocyte size was expressed relative to heart weight, the relative size of cardiomyocytes was greater in PR fetuses compared to Controls. Whilst this might suggest that there are fewer but relatively larger cardiomyocytes present in the heart of the growth restricted fetus, further studies are required to determine the impact of placental restriction on the number of cardiomyocytes in the heart of the PR fetus. Acute placental restriction induced by uteroplacental embolization did not alter cardiomyocyte size (6, 26). This is likely to be due to the difference in duration and degree of fetal hypoxemia, and fetal neuroendocrine responses in the embolisation model compared to the chronic placental restriction (PR) model.
of fetal growth restriction. There are a series of neuroendocrine responses to the decrease in fetal substrate supply in the PR fetus. Such responses include increased plasma cortisol concentrations (37), decreased plasma IGF-1 and IGF-2 concentrations (36) and increased circulating plasma noradrenaline concentrations (42). There is conflicting evidence for a role for cortisol in determining the normal profile of cardiomyocyte maturation or growth in late gestation. Intrafetal infusion of cortisol to levels which mimic the prepartum rise result in an increase in cardiomyocyte proliferation after 2d of infusion (~7nmol/l) (17). In contrast, infusion of cortisol at levels that result in supraphysiological fetal plasma cortisol concentrations (~1000nM) and fetal hypertension led to an increase in cardiomyocyte size (28). In the present study there was no relationship between fetal plasma cortisol concentrations and any cardiomyocyte parameter measured, which may suggest that there are factors other than cortisol in the growth restricted fetus which determine the growth profile of the cardiomyocytes within the heart. Further investigation of the mechanisms underlying the altered development of the cardiomyocyte population in the heart of the PR fetus may provide new insights into the mechanisms regulating the transition from hyperplastic to hypertrophic heart growth.

IUGR as a result of chronic hypoxia or maternal protein restriction in rats caused an increase in apoptosis of cardiomyocytes (3) and a reduction in cardiomyocyte number at birth (10) respectively. Although we did not directly measure the number of cardiomyocytes present in the fetal heart of the IUGR fetus, the possibility remains that there is an overall decrease in the number of cardiomyocytes in the heart of the PR fetal sheep. A decrease in cardiomyocyte number is supported by our finding that relative cardiomyocyte size is greater in PR fetuses and would result in an increase in postnatal hypertrophic growth (27) in order to maintain heart growth. This could be one mechanism which results in an increased susceptibility to cardiac injury during adult life (25).
In summary we have shown that restriction of placental growth in the sheep results in chronic fetal hypoxia, fetal growth restriction, an increase in the proportion of mononucleated cardiomyocytes and cardiomyocytes that are smaller in absolute terms but larger relative to heart size. These changes in the fetal heart may be an adaptation to chronic hypoxia which results in fetal growth restriction associated with more mononucleated cardiomyocytes, a delay in binucleation and relatively larger cardiomyocytes in postnatal life. These findings are consistent with a reduction in cardiomyocyte number in the heart of the growth restricted fetus. There may be a neonatal period where interventions may normalise cardiomyocyte number in the heart and lead to a reduction in the adult vulnerability to heart disease.
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REFERENCES


Table 1. Number of animals in experimental groups.

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<tr>
<td></td>
<td>Control</td>
<td>PR</td>
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<tr>
<td>No. of Fetuses</td>
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<td>No. of Singles</td>
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<td>4</td>
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<td>No. of Twins</td>
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<td>No. of Females</td>
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<td>No. of Males</td>
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Mean ± SEM. No., Number
Table 2. Characteristics of fetuses at post mortem.

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<tr>
<td></td>
<td>Control (n=9)</td>
<td>PR (n=4)</td>
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<tr>
<td>Gestational Age (d)</td>
<td>132.8 ± 0.3</td>
<td>133.2 ± 0.2</td>
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<tr>
<td>Fetal Body Weight (kg)</td>
<td>3.8 ± 0.3</td>
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<tr>
<td>Heart Weight (g)</td>
<td>32.4 ± 3.1</td>
<td>17.6 ± 2.0 *</td>
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<tr>
<td>Relative Heart Weight (g/kg)</td>
<td>8.4 ± 0.5</td>
<td>7.9 ± 0.4</td>
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<td>Number of Fetuses</td>
<td>1.5 ± 0.2</td>
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Mean ± SEM. 2 way ANOVA; treatment (Control, PR) and age (<135d, >135d); *, treatment effect; #, age effect, P<0.05.
Table 3. Mean gestational blood gas and pH values.

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<td>Control (n=7)</td>
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<td>PO₂ (mmHg)</td>
<td>20.8 ± 0.5</td>
<td>14.5 ± 1.6 *</td>
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<td>Oxygen Saturation (%)</td>
<td>68.2 ± 2.4</td>
<td>43.2 ± 6.0 *</td>
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<tr>
<td>Oxygen Content (ml/dl)</td>
<td>8.6 ± 0.4</td>
<td>6.3 ± 0.5 *</td>
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<tr>
<td>PCO₂ (mmHg)</td>
<td>44.3 ± 0.9</td>
<td>48.5 ± 0.9 *</td>
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<tr>
<td>pH</td>
<td>7.376 ± 0.015</td>
<td>7.377 ± 0.016 *</td>
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<tr>
<td>Hemoglobin (g/dl)</td>
<td>9.1 ± 0.5</td>
<td>11.0 ± 1.5</td>
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Mean ± SEM. 2 way ANOVA; treatment (Control, PR) and age (<135d, >135d); *, treatment effect; #, age effect, P<0.05.
### Table 4. Length and width of mononucleated cardiomyocytes isolated from hearts of Control and PR fetuses in <135d and >135d groups.

<table>
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<tbody>
<tr>
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<td>Control (n=9)</td>
<td>PR (n=4)</td>
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<tr>
<td><strong>Mononucleated Cardiomyocytes</strong></td>
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<td></td>
</tr>
<tr>
<td>Length (µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Ventricle</td>
<td>68.1 ± 1.8</td>
<td>64.8 ± 4.4 *</td>
</tr>
<tr>
<td>Left Ventricle</td>
<td>64.8 ± 1.0 Ψ</td>
<td>63.1 ± 3.8 * Ψ</td>
</tr>
<tr>
<td>Width (µm)</td>
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<tr>
<td>Right Ventricle</td>
<td>10.8 ± 0.3</td>
<td>10.4 ± 0.5 *</td>
</tr>
<tr>
<td>Left Ventricle</td>
<td>10.0 ± 0.4 Ψ</td>
<td>9.4 ± 0.4 * Ψ</td>
</tr>
<tr>
<td><strong>Binucleated Cardiomyocytes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length (µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Ventricle</td>
<td>89.3 ± 1.9</td>
<td>91.4 ± 4.1</td>
</tr>
<tr>
<td>Left Ventricle</td>
<td>85.8 ± 1.3 Ψ</td>
<td>84.7 ± 4.8 Ψ</td>
</tr>
<tr>
<td>Width (µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Ventricle</td>
<td>11.6 ± 0.3</td>
<td>10.7 ± 0.3 *</td>
</tr>
<tr>
<td>Left Ventricle</td>
<td>10.8 ± 0.2 Ψ</td>
<td>10.1 ± 0.3 * Ψ</td>
</tr>
</tbody>
</table>

Mean ± SEM. 3 way ANOVA; treatment (Control, PR); age (<135d, >135d); ventricle (right, left). *, treatment effect; #; age effect; Ψ, ventricle effect, P<0.05.
FIGURE LEGENDS

Figure 1. A. There is a higher percentage of mononucleated cardiomyocytes in the ventricles of PR (open hatches) compared to Control fetuses (closed hatches; 3 way ANOVA; treatment (Control, PR; \( P=0.001 \)), age (<135d, >135d), ventricle (right, left)). B. There is a significant relationship between mean gestational arterial PO2 and the percentage of mononucleated cardiomyocytes in the right (\( P<0.01, r^2=0.272, \%=-1.15 \ PO_2 + 68.91 \)) and left ventricle (\( P<0.001, r^2=0.475, \%=-2.14 \ PO_2 + 88.03 \)). Circles, <135d; Triangles, >135d; Filled, PR; Open, control.

Figure 2. A, Representative image of a methylene blue stained mononucleated cardiomyocyte (1) and a Ki67+ (DAB visualised) mononucleated cardiomyocyte (2). B, There is no difference in the percent of mononucleated cardiomyocytes positive for Ki67 in the ventricles of the PR (closed bars) compared to the Control (open bars) fetuses in the right and left ventricle at <135d and >135d gestation (term, 150 d).

Figure 3. Cardiomyocyte size measures expressed relative to heart weight in Control (open bars) and PR (closed bars) fetuses in the right and left ventricle at <135d and >135d gestation (term, 150d) (Panels A-D). Mononucleated and binucleated cardiomyocytes are longer (mononucleated, \( P<0.01 \); binucleated, \( P<0.01 \)) and wider (mononucleated, \( P<0.01 \); binucleated, \( P<0.01 \)) relative to heart size in PR fetuses compared to Control fetuses (indicated by *). With increasing gestational age, cardiomyocytes become more narrow (mononucleated, \( P<0.05 \); binucleated, \( P<0.05 \); as indicated by #). In both the
right (RV, solid line) and left (LV, dashed line) ventricle there is a direct relationship between mean gestational PO₂ and binucleated cardiomyocyte size (RV (solid line), $r^2=0.683$, $P<0.01$, length=$8.9–0.271*PO₂$, LV (dashed line), $r^2=0.668$, $P<0.01$, length=$8.157-0.244*PO₂$; RV (solid line), $r^2=0.629$, $P<0.01$, width=$1.038–0.031*PO₂$, LV (dashed line), $r^2=0.646$, $P<0.01$, width=$0.932–0.027*PO₂$; Panels E and F)
Figure 1
Figure 2
Figure 3