Author/s:
Sutherland, MR;Ng, KW;Drenckhahn, JD;Wlodek, ME;Black, MJ

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Black Mary Jane (Orcid ID: 0000-0002-9253-3773)

Impact of Intrauterine Growth Restriction on the Capillarisation of the Early Postnatal Rat Heart

Megan R. Sutherland[#1], Ka Wing Ng[#1], Jörg D. Drenckhahn[2], Mary E. Wlodek[3], and Mary Jane Black[1]

[1] Department of Anatomy and Developmental Biology and the Biomedicine Discovery Institute, Monash University, Clayton, Victoria, Australia

[2] Department of Pediatric Cardiology, Justus Liebig University Giessen, Giessen, Germany

[3] Department of Physiology, School of Biomedical Sciences, University of Melbourne, Melbourne, Victoria, Australia

[#]Authors Megan R. Sutherland and Ka Wing Ng contributed equally

Corresponding Author:

Professor M. Jane Black
Department of Anatomy & Developmental Biology
19 Innovation Walk, Building 76
Monash University
Clayton, Victoria
3800, Australia
Ph: +61 3 9902 9112
email: jane.black@monash.edu

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ABSTRACT

Capillarisation plays a key role in the growth of the developing heart. We therefore hypothesised that impaired heart development following intrauterine growth restriction (IUGR) may arise from inadequate myocardial capillary growth. The aims of the study were to examine the effect of IUGR on the growth and diffusion radius of intramyocardial capillaries in rats at postnatal day 1.

Uteroplacental insufficiency was induced in rats in late gestation (E18, term=E22) by bilateral uterine artery and vein ligation (Restricted offspring n=12; 6 males and 6 females); offspring from sham-operated dams were used as Controls (n=10; 5 males and 5 females). At postnatal day 1, the hearts were immersion-fixed and heart volume, capillary length density, capillary diffusion radius and total capillary length were stereologically determined.

Restricted offspring were significantly smaller at birth, with a concomitant reduction in heart volume and total myocardial capillary length compared to Controls. Capillary growth was not impaired relative to heart size, with no significant differences in capillary length density or diffusion radius in the myocardium of Restricted and Control offspring. There were no sex differences in any of the parameters examined.

In conclusion, there was no evidence to indicate that microvascular development is compromised in the heart of IUGR offspring at one day after birth. Total myocardial capillary length, however, was significantly reduced in the growth restricted offspring and further

longitudinal studies are required to elucidate the long-term impact, particularly following hypertrophic cardiac growth.

Key words: angiogenesis, heart development, fetal growth restriction, organ size, cardiovascular disease.

INTRODUCTION

Many epidemiological studies have demonstrated a link between low birth weight and an increased risk of ischemic heart disease later in life (Barker et al., 1989; Eriksson et al., 1999; Huxley et al., 2007; Andersen et al., 2010) The underlying mechanisms, however, are poorly understood. Low birth weight (< 2,500 g) affects approximately 15% of newborn infants worldwide (United Nations Children's Fund and World Health Organization, 2004), and in term-born infants is the consequence of intrauterine growth restriction (IUGR). IUGR results from a reduced oxygen and nutrient supply to the developing fetus, for which there are numerous causes including maternal undernutrition and impaired placental function (Nardozza et al., 2017). Late gestational uteroplacental insufficiency is the leading cause of IUGR in developed countries (Henriksen and Clausen, 2002; Resnik, 2002).

Cardiac growth, and ultimately the size of the heart in the developing fetus, is the result of coordinated growth of both the cardiomyocytes and the coronary vasculature and is therefore dependent on angiogenesis (Tomanek et al., 1999; Tirziu et al., 2007). Conversely, inadequate growth of the coronary vasculature can adversely impact blood supply to the myocardium and affect cardiac growth (Shiojima et al., 2005; Rhee et al., 2018). Hence, the

reported reduction in the complement of cardiomyocytes in the hearts of IUGR offspring (Corstius et al., 2005; Black et al., 2012; Botting et al., 2014) may be the consequence of attenuated myocardial capillary growth. In this regard, capillary rarefaction (reduced density of capillaries) has previously been described in a number of vascular beds (including the heart) in experimental studies of fetal and newborn IUGR offspring (Boujendar et al., 2003; Khorram et al., 2007; Rozance et al., 2011; Liu et al., 2014; Rozance et al., 2015; Schipke et al., 2017). In postnatal life, the increased oxygen requirement of cardiac muscle, particularly if cardiac hypertrophy develops, requires the tissue to be highly vascularised (Oka et al., 2014), with endothelial cells (comprising the capillaries) the major non-muscle cellular constituent of the heart (Pinto et al., 2016). Therefore, if myocardial capillary rarefaction is present following IUGR this has the potential to contribute to the long-term vulnerability to coronary ischemia, especially given that the capacity for cardiac angiogenesis is substantially diminished in adulthood (Weinsaft and Edelberg, 2001).

To date, the findings of previous studies examining the impact of IUGR on myocardial capillarisation at the beginning of life are conflicting. In a sheep uterine carunculectomy model, increased capillary density was reported in the right ventricle of growth restricted fetal lambs (mixed sex; left ventricle not assessed) (Botting et al., 2014), whereas at 3 weeks of age a significant reduction in total capillary length in the left ventricle was observed (mixed sex; right ventricle not assessed, and capillary density was not reported) (Wang et al., 2015). In newborn male rat pups exposed to maternal protein restriction throughout gestation, there was no effect on myocardial capillary density (Menendez-Castro et al., 2014). Most recently, however, rabbit fetuses (of mixed sex) affected by late-gestation

uteroplacental insufficiency were found to exhibit capillary rarefaction in the left ventricle, but not in the right ventricle (Schipke et al., 2017). Differences in species and the model of IUGR likely contribute to some of these differences in findings.

To explore this further, in this study we utilised a rat model (where the spatial and temporal development of the coronary vasculature is well-characterised (Ratajska and Fiejka, 1999; Ratajska et al., 2003)) to examine the effect of uteroplacental insufficiency, that was induced during late gestation (at E18, term=E22), on myocardial capillarisation. This is a critical period of myocardial capillary development in the rat, where following connection with the systemic circulation at E16, the capillary network develops *in utero* from a thin disorganised layer to a highly organised microvascular network extending through the full thickness of the developing myocardium (Ratajska et al., 2003). In our study, IUGR was induced in rats by bilateral uterine vessel ligation and the hearts of both male and female offspring were analysed, as it is now well-recognised that there is sexual dimorphism in the programming effects of growth restriction on the cardiovascular system in animal studies (Ojeda et al., 2007a; Ojeda et al., 2007b; Wlodek et al., 2007; Wlodek et al., 2008; Moritz et al., 2009; Wadley et al., 2010; Wadley et al., 2013; Gallo et al., 2014; Intapad et al., 2014; Cheong et al., 2016), and in some human studies (Huxley et al., 2007; Dasinger and Alexander, 2016) .

MATERIALS AND METHODS

Animal studies and the induction of growth restriction

All animal experiments were approved by The University of Melbourne Pharmacology, Physiology, Biochemistry and Molecular Biology and Bio21 Institute Animal Ethics Committee (0004138), and the treatment and care of the animals adhered to the *Australian code for the care and use of animals for scientific purposes*. Wistar-Kyoto rats were housed in a temperature-controlled environment with a 12:12h light-dark cycle, and received standard food pellets and water *ad libitum*. Uteroplacental insufficiency (Restricted) was induced in pregnant dams (9-13 weeks of age) by bilateral uterine artery and vein ligation at day 18 of gestation (term is 22 days), as previously described (Wlodek et al., 2005; Wlodek et al., 2008; Moritz et al., 2009). Briefly, rats were anaesthetised (for a duration of 40 min) with an intravenous injection of 50mg/kg ketamine (Parnell Laboratories, NSW, Australia) and 10 mg/kg Ilium Xylazil-20 (Troy Laboratories, Smithfield, NSW, Australia). Under aseptic conditions, a midline abdominal incision was made to expose the cervical end of the uterus, and the left and right uterine vessels were ligated (surgery duration of approximately 10 min). Sham-surgeries were conducted in the dams of the Control group. The Restricted offspring (n = 12; 6 males and 6 females) and Control offspring (n = 10; 5 males and 5 females) were spontaneously delivered at term (day 22), then were weighed and humanely killed the day after birth (postnatal day 1, PN1). Only 1 male pup and 1 female pup (randomly selected) were analysed from each litter; sex was determined by visual examination of the anogenital distance. At necropsy, the hearts were excised and immersion-fixed in 10% buffered formalin. Prior to analysis, large vessels were removed and the hearts were weighed. Researchers were blinded to the experimental grouping of the animals for all

subsequent analyses.

Assessment of heart volume

Due to the anisotropic nature of the myocardial capillaries, the hearts were each cut in half according to Systematic Version 1 of the Orientator method (Mattfeldt et al., 1990), thus generating isotropic sections of the myocardial vasculature. The tissue was then processed and embedded in paraffin, with both pieces of each heart embedded in the same block. When embedding the tissue, the angled cut surfaces were carefully positioned so that they were flush with the top of the blocks. Blocks were serially sectioned at 5 µm, and commencing with a random number between 1 and 10, every 10^{th} and 11^{th} sections were collected.

Every 10^{th} section from each heart was stained with haematoxylin. Images of the haematoxylin-stained heart sections were acquired using ImagePro Plus software (version 6.2; Media Cybernetics, Rockville, MD, USA); an orthogonal grid was superimposed over the images, and the number of intersecting grid points overlaying heart tissue were counted. Heart wall volume (V_{heart}) was then calculated using the Cavalieri principle (Gundersen and Jensen, 1987):

$$V_{heart}(mm^3) = {}^{1}/_{F1} \times T \times a(p) \times P_{tissue}$$

Where F1 is the sampling fraction (1/10) as every 10^{th} section was analysed; T is the section thickness (0.005 mm), a(p) is the area associated with each grid point on the orthogonal grid, and P_{tissue} is the total number of counted grid points overlaying the heart wall.

Assessment of cardiac capillarisation

Myocardial capillaries were fluorescently labelled in every 30th section of heart (beginning from the 11th section) with isolectin *Griffonia simplicifolia*-IB4 (GS-IB4). Sections underwent heat-mediated antigen retrieval in Tris-EDTA buffer and blocking in CAS-Block (Life Technologies, Frederick, MD, USA), prior to incubation for 1 hour at room temperature with Alexa Fluor 568-conjugated isolectin GS-IB4 at a 1:50 dilution (I21412; Molecular Probes, Life Technologies, Eugene, OR, USA). Stained sections were viewed using a 40x lens, and were systematically sampled in the X and Y directions at a step length of 445 µm. At each field of view (18 – 28 fields of view per section) images were acquired using cellSens Entry software (version 1.15; Olympus Corporation, Tokyo, Japan). An orthogonal grid within an unbiased counting frame was superimposed over each image, and the number of intersecting grid points overlaying the myocardium (P_{tissue}), and the number of capillary profiles within the counting frame (Q-), were counted (Figure 1). Capillary length density (Lv_{cap}), the length of capillaries per unit volume of heart wall, was then calculated using the following formula (Mattfeldt et al., 1990; Lim et al., 2006):

$$Lvcap\ \left({}^{mm}/_{mm^3}\right) = \frac{(2\ \times\ Q^-)}{P_{tissue}\ \times\ a(p)}$$

To determine total capillary length per heart, capillary length density (Lv_{cap}) was multiplied by heart wall volume (V_{heart}). Capillary diffusion radius (r), a measure of average maximal diffusion distance from capillary to tissue, was then calculated using the following formula (Lim et al., 2006; Tang et al., 2009):

$$r\ (mm) = \sqrt{\frac{1}{\pi \times Lvcap}}$$

Statistical analysis

Statistical analyses were performed using Graphpad Prism (version 7.02; Graphpad Software Inc, La Jolla CA, USA). Litter size was analysed using an unpaired two-tailed Student's t test. All other data were analysed using a two-way analysis of variance (ANOVA), with restriction (p_{Rest}), sex (p_{Sex}) and their interaction ($p_{Rest*Sex}$) as factors; this was followed by a Tukey's post-hoc test. Data are reported as the mean ± the standard error of the mean (SEM). Statistical significance was accepted at $p < 0.05$. It is to be noted that due to a technical error during sectioning, one of the female controls was not included in the analysis of heart volume and total capillary length.

RESULTS

Litter and offspring size at birth

Litter size at birth for the Restricted litters with maternal bilateral uterine vessel ligation was significantly less than in the sham-operated Controls (Table 1). Restricted pups were significantly smaller than Controls (Table 1), with a reduction in both body weight ($p = 0.0002$) and crown rump length ($p = 0.0008$) at PN1. There were no differences between the sexes in body weight or crown rump length, and no significant interaction effect between sex

and growth restriction.

Heart weight and heart wall volume

In accordance with the differences in body weight and CRL, there was a significant reduction in both heart weight (Table 1) and heart wall volume (Figure 2) in the Restricted offspring compared to Controls. The reduction in heart weight and heart wall volume was proportional to the decrease in body size in the Restricted group, with no significant differences in heart weight (Table 1) or heart wall volume (Figure 2) between the groups when adjusted for body weight. There were no differences between the sexes in relation to absolute or relative heart weight (Table 1) or heart wall volume (Figure 2).

Myocardial capillarisation

Capillary diffusion radius and length density of the myocardial capillaries in the Restricted and Control offspring are shown in Figure 3A and 3B, respectively; total capillary length per heart is shown in Figure 3C. There were no significant differences in the length density or in the diffusion radius of the capillaries in the myocardium of the Restricted and Control offspring; however, the overall total capillary length per heart was significantly reduced ($p = 0.002$) in the Restricted group. The sex of the offspring had no effect on myocardial capillarisation, and there was no difference between the sexes in myocardial capillarisation following uteroplacental insufficiency.

DISCUSSION

In this study there was no evidence of impaired capillarisation of the myocardium after birth in growth restricted rat offspring following the induction of uteroplacental insufficiency in late gestation, with the length density and diffusion radius of myocardial capillaries not different to Control offspring. There was, however, a significant reduction in the total length of myocardial capillaries in the Restricted offspring, given that they had proportionally smaller hearts (likely due to a reduction in cardiomyocyte endowment (Black et al., 2012)). Although it is known that the early life environment can lead to different cardiovascular programming effects in males and females (Huxley et al., 2007; Ojeda et al., 2007a; Ojeda et al., 2007b; Wlodek et al., 2007; Wlodek et al., 2008; Moritz et al., 2009; Wadley et al., 2010; Wadley et al., 2013; Gallo et al., 2014; Intapad et al., 2014; Cheong et al., 2016; Dasinger and Alexander, 2016), in this study we found no differences between sexes in any of the experimental parameters examined.

Prior to the commencement of this study, reduced vascular endothelial growth factor expression and/or reduced capillary growth had been described in a number of major organs in association with growth restriction *in utero* (Boujendar et al., 2003; Pladys et al., 2005; Khorram et al., 2007; Ham et al., 2009; Rozance et al., 2011; Liu et al., 2014; Rozance et al., 2015); hence, it was conceivable that the capillarisation of the heart would also be adversely affected. However, previous findings in relation to the effects of growth restriction on fetal/newborn myocardial capillarisation (with studies conducted in three different animal

models) have been equivocal, with reports of increased capillary density (lamb fetuses, only the right ventricle was examined) (Botting et al., 2014), no change in capillary density (rat studies, whole heart) (Menendez-Castro et al., 2014), or capillary rarefaction (rabbit studies, with reduced capillary density in the left ventricle but not in the right ventricle) (Schipke et al., 2017).

Given that the temporal and spatial development of the coronary vasculature in the rat heart is relatively well described (Ratajska and Fiejka, 1999; Ratajska et al., 2003), the rat was considered a good model to determine the impact of late gestational uteroplacental insufficiency on the developing heart. Importantly, we found no adverse effects on capillary density or capillary diffusion radius within the myocardium of Restricted rat offspring at postnatal day 1, thus suggesting that the overall blood supply to the cardiac muscle was not compromised. Using our methodological approach where the whole heart was embedded, we could not separately examine capillarisation in the right and left ventricles. Our findings are in accordance with the previous study conducted in rats at the same time point, where no effects on myocardial capillary density were observed in the hearts of male rat pups at postnatal day 1 when growth restriction had been induced by maternal low protein diet (LPD) throughout pregnancy (Menendez-Castro et al., 2014). Taken together, the combined findings in these rat models suggest that whether growth restriction is induced over a chronic time course, due to maternal malnutrition (common cause of IUGR in developing countries) or late in gestation due to uteroplacental insufficiency (common cause of IUGR in developed countries), the density of myocardial capillaries remains unchanged, with capillary growth directly proportional to heart size. Our findings are also supported by that of Wang et al.

(2015), who reported a significant reduction in total capillary length in the left ventricle of 3 week old growth-restricted lambs, which is consistent with their smaller ventricular volume.

Whether capillary growth dictates heart size (and potentially body size) or *vice versa* could not be determined in these studies. Indeed, the reciprocal relationship between myocardial angiogenesis and cardiac growth has previously been highlighted in experimental studies utilising a transgenic mouse model with regulatable expression of the angiogenic growth factor PR39 in the cardiomyocytes. In that model, an increase in endothelial cell mass in the developing heart was shown to drive an increase in cardiac size in the absence of hemodynamic or any other known hypertrophic stimulus (Tirziu et al., 2007). Tomanek et al. (1999) has also previously shown in an embryonic chicken model that the level of capillary growth remains proportional to cardiac growth in the developing heart when there is either an increase or decrease in cardiac mass, which further supports our findings of proportional heart size and capillary length in all offspring.

The long-term impact of growth restriction on myocardial capillary density has, to our knowledge, only been investigated in maternal LPD rat models, in the absence of postnatal catch-up growth and cardiac hypertrophy. Menendez-Castro *et al*. (2014) reported no effect on myocardial vessel density in LPD rats at PN1, however a significant increase in density was evident at 10 weeks of age. Conversely, we have shown that total myocardial capillary length and surface area, and density, is unaffected in growth restricted (LPD) rats at 24 weeks of age (Lim et al., 2006). Further longitudinal studies are therefore required to fully assess the long-term impact of growth restriction on myocardial capillarisation. Indeed, with ageing the capacity for myocardial angiogenesis is known to diminish (Rakusan et al., 1992; Flanagan et

al., 1994; Hudlicka and Brown, 1996; Edelberg et al., 2002). As such, the smaller coronary vascular tree in the growth restricted heart at the beginning of life (with significantly reduced overall capillary length) may not be able to adequately support hypertrophic growth of the heart in later life, particularly in cases where physiological or pathological cardiac hypertrophy develops (as we have previously reported in this uteroplacental insufficiency model (Wlodek et al., 2008; Wadley et al., 2016)). It is therefore conceivable that the heart may not be able to undergo sufficient angiogenesis to maintain capillary density, which would therefore adversely impact myocardial blood supply. This would provide a plausible explanation for the increased risk of ischemic heart disease in adults born growth restricted (Barker et al., 1989; Eriksson et al., 1999; Huxley et al., 2007; Andersen et al., 2010), especially when there is postnatal catch-up body growth (Eriksson et al., 1999; Andersen et al., 2010). This is an important area for future research.

In conclusion, the findings of this study suggest that late gestational growth restriction does not adversely impact microvascular development in the myocardium, as there was no effect of growth restriction on the length density or diffusion radius of the myocardial capillaries after birth. Total myocardial capillary length, however, was significantly reduced in the growth restricted offspring. Further longitudinal studies are required to fully elucidate the long-term impact of growth restriction on the myocardial vasculature, particularly in cases of physiological and pathological hypertrophy.

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FIGURE LEGENDS

Figure 1: Representative image of isolectin-labelled capillaries in the rat myocardium at postnatal day 1, overlayed by an orthogonal grid (thin lines) and unbiased counting frame (thick lines). Capillary profiles within the unbiased counting frame, or touching the inclusion lines (dashed) were counted, whereas those on the exclusion lines of the frame (solid) were excluded. Any branches from a capillary profile were counted individually. Tiny dots not recognisable as a full capillary, and any large vessels, were excluded. Bar = 50 μm.

Figure 2: Absolute heart volume (A) and heart volume relative to body weight (B) in Control (n=5 male and 4 female) and Restricted (n=6 male and 6 female) rat pups at postnatal day 1. Analysed by two-way ANOVA with the factors growth restriction (p_{Rest}), sex (p_{Sex}) and their interaction ($p_{Rest*Sex}$).

Figure 3: Myocardial capillary diffusion radius (A) and capillary length density (B) in Control (n=5 male and 5 female) and Restricted (n=6 male and 6 female) rat pups at postnatal

day 1. Total capillary length (C) in Control (n=5 male and 4 female) and Restricted (n=6 male and 6 female) rat pups at postnatal day 1. Analysed by two-way ANOVA with the factors growth restriction (p_{Rest}), sex (p_{Sex}) and their interaction ($p_{Rest*Sex}$).