Does prenatal hypoxia lead to permanent cardiovascular change in the offspring?

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Abstract

Chronic prenatal hypoxia is associated with intrauterine growth retardation and there is now some evidence that it also induces programmed hypertension in offspring. However these studies are generally confounded as hypoxia is either induced by maternal hypoxia or placental insufficiency. The study described in this thesis is designed to overcome this problem. Pregnant rats were dosed daily with the drug dofetilide (2.5 mg/kg) or water on GD 11-14 and the cardiovascular parameters of the offspring at 8-12 weeks (>300g) were analysed using implanted telemetry blood pressure (BP) transmitters.

Dofetilide is a class III antiarrhythmic drug that selectively blocks the \( I_{kr} \) channel which is expressed in the rat embryo but not in the adult rat. When administered to pregnant rats it induces bradycardia (and associated hypoxia) in the embryos without affecting maternal oxygenation or heart rate. Embryo culture studies showed that dofetilide induced a period of embryonic bradycardia for up to 9 hours following each dose. The dofetilide treated-rats had less completed pregnancies, smaller litters and lower weight pups compared to controls. At 8-12 weeks age the dofetilide offspring has increased BP (+10-12%) compared with controls. Postnatal stress in the form of air puffs did not reveal other cardiovascular differences between control and dofetilide offspring. The increased BP was not associated with an increased HR or changes in the autonomic nervous system. Remaining unexplored possibilities include impaired nephrogenesis, vascular dysfunction and microvascular rarefaction.
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1. Introduction

The most common cause of death in Australia is cardiovascular disease (AIHW, 2010). Hypertension is one of the major risk factors for cardiovascular diseases and renal failure. The higher the blood pressure (BP), the greater is the risk to develop these diseases.

1.1. Blood pressure regulation

Blood pressure is controlled by the autonomic nervous system (ANS) and is regulated via changes in sympathetic and parasympathetic activity. Homeostatic mechanisms control blood pressure so that it meets the requirement of the tissues need for nutrients and oxygen while remaining low enough to avoid structural damage to the vasculature and vital organs (Dampney et al., 2002, Boron and Boulpaep, 2012, Martini et al., 2012).

Blood pressure regulation is constantly monitored by the baroreceptor reflex which is one of the most important homeostatic reflex mechanisms. The monitoring is performed via baroreceptors which are mechanoreceptors located in the carotid sinus and the aortic arch. The receptor signal changes with changing BP and the information is transmitted to the cardio regulatory centers in the brain. If the BP increases, the arteries stretch and the baroreceptor firing rate increases. This leads to a decrease in sympathetic activity and an increase in parasympathetic activity to return the BP to appropriate level. A decrease in sympathetic activity will lead to decreased heart rate (HR) and stroke volume which in turn will lead to decreased arterial pressure (Dampney et al., 2002).

In hypertensive patients, heart rate is either normal, or frequently found to be elevated. If the reflex was functioning appropriately, bradycardia (slow heart rate) would be a characteristic feature of hypertension, since the elevation in blood pressure should produce reflex decreases in HR. As heart rate is generally normal, this implies either a primary dysfunction, or resetting of the baroreflex to a higher set-point around which blood pressure is regulated (Bristow et al., 1969).
1.2. **Risk factors for hypertension**

The risk factors for hypertension include both lifestyle factors that can be controlled and biomedical factors such as genetic and epigenetic that cannot be controlled. The most likely cause of hypertension is believed to be a combination of environmental and genetic factors where the interaction causes a change in gene expression. These factors are known as epigenetic factors (Millis, 2011). If this occurs during gestation, it can have consequences that persist throughout life (Thornburg et al., 2010). This concept is known as developmental programming.

1.3. **Developmental programming**

The phenomenon developmental programming was first reported in a series of epidemiologic studies of cardiovascular risk lead by David Barker approximately 20 years ago (Barker, 1996). In these studies a link between low birth weight and an increased risk of a variety of diseases in later life, including obesity, type 2 diabetes, myocardial infarction, and systolic hypertension was identified (Barker et al., 1993, Barker, 1992, Eriksson et al., 2000, Barker et al., 2002). Barker went on to report an inverse relationship between birth weight and BP (Barker and Osmond, 1988).

Similar results have been observed in animal studies. The BP in low birth weight rats is significantly higher compared to pups from the same litter with normal weight (Vuguin, 2007). A number of in utero stressors have been shown to program hypertension in animals. These include maternal low calorie diet, maternal low protein diet, maternal high gestational salt diet, maternal high fat diet, high gestational glucocorticoid and maternal hypoxia (Vuguin, 2007, Alexander and Tuan, 2010).

1.4. **Animal models of programmed hypertension using Hypoxia**

Hypoxia can result from a number of different factors such as local vascular disease, chronic lung or cardiovascular disease or high altitude exposure (Jakoubek et al., 2008) Placental dysfunction due to placental insufficiency, cord compression, preeclampsia can also reduce oxygen delivery to the embryo (Chen and Zhang, 2011). The protocol to induce embryonic hypoxia in mammals generally involves placing pregnant rats in a hypoxic chamber at 10% to 12% O₂ through gestation (Coney and Marshall, 2010, Hauton and Ousley, 2009).
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However, the various *in utero* stressors described above all have the confounding feature that the pregnant animal, as well as the embryos are exposed to the stress. Hence the maternal stress response is an additional parameter to be considered in interpreting the outcome of the studies. The study described in this thesis is designed to overcome this problem.

1.5. **Dofetilide effects on the developing embryo**

In the present study pregnant rats were exposed to the drug dofetilide on four days of gestation (GD 11, 12, 13 and 14) out of a total gestational period of 22 days. Dofetilide is a class III antiarrhythmic that selectively inhibits a potassium current called the rapid inward rectifying current ($I_{Kr}$) in the heart (Ficker et al., 1998, Srivastava et al., 2005). The rat embryonic heart starts to beat on GD 10 and for the next 4 days (GD 11-14) is highly dependent on the potassium current $I_{Kr}$ for normal repolarisation of the action potential (Abrahamsson et al., 1994, Spence et al., 1994). Both *in vitro* and *in vivo* studies have shown that dofetilide induces a concentration-dependent slowing of the HR of exposed rat embryos (Webster et al., 1996, Nilsson et al., 2013, Ritchie et al., 2013). If the concentration is too high the heart stops and the embryos die (Abela et al., 2010). After GD 15 repolarisation involves other potassium currents and the fetal heart is no longer affected by dofetilide (Nilsson et al., 2013).

With increasing gestational age, other currents continue to dominate such that the newborn and adult rat hearts are not dependent on $I_{Kr}$ for their normal functioning (Nerbonne, 2004, Abrahamsson et al., 1994). It has been shown that the period of bradycardia induced by a single dose of dofetilide persists for at least 6 hours and causes the embryos to become hypoxic for at least 4 hours (Ritchie et al., 2013). The animal model developed for this study involves dosing pregnant rats with dofetilide using a dose that slows the embryonic heart for a number of hours (thus inducing hypoxia) on each treatment day (GD 11, 12, 13 and 14) without affecting maternal oxygenation or heart rate.

1.6. **Aims and Hypothesis**

The project has two main aims. The first is to confirm the effect of dofetilide on the embryonic heart rate. The second is to determine the effects of prenatal hypoxia on cardiovascular and autonomic parameters in the young adult rat.
2. Materials and methods

2.1. Animals

The use of animals in this study was approved by the Animals Ethical Review Committee at the University of Sydney. Male and female Sprague-Dawley rats were mated overnight and the next morning the vaginal smear of the females were examined for sperm to confirm pregnancy (GD 0). The male rats were then returned to their cages while the mated females were kept in cages together.

2.2. Embryonic heart rate and rhythm

2.2.1. Embryo culture

In order to determine the effect of dofetilide on embryonic heart rate, 8 female rats were dosed with 2.5 mg/kg dofetilide or water once a day from GD 11. On GD 11, 12, 13 or 14, the rats were anaesthetized by CO₂ inhalation and euthanized by cervical dislocation (Table 1). Embryos were collected 2 or 9 hours after dosing on each GD with one control rat and one dofetilide rat sacrificed at each time point. At least 5 embryos from each dam were collected for embryo culture. Embryos were cultured two or nine hours after the last dose.

Table 1: Embryo culture schedule. Dofetilide exposed and control rats were dosed once a day from GD 11. Embryos were cultured on GD 11, 12, 13 or 14, 2 or 9 hours after the last dose.
The intact uterus was briefly rinsed with phosphate buffered saline (PBS) (Sigma) and implantations were counted and then removed. The decidua and the Reichert’s membrane of the embryo were removed using forceps under a dissecting microscope. An incision was carefully made in the yolk sac away from major blood vessels. The embryo was then pushed through the incision while yolk sac blood vessels remained intact and the amnion was opened. The embryos were placed in separate culture bottles containing 2.5 ml of Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St. Louis, USA). The culture bottles were placed in a rotating culture system (B.T.C. Engineering, Milton, Cambridge, England) which rotated 30 times per minute. The bottles were continuously gassed with 5% CO$_2$ and 95% O$_2$ at 37°C. After 15 minutes equilibration, the embryo, still within their bottles, were individually placed under a dissecting microscope (Leica M420 Leica Microsystems Ltd, Heerbrugg, Switzerland) fitted with a video recorder (Olympus DP70, Olympus Australia, Pty, Melbourne, Australia). To maintain the temperature of the embryos, the microscope was equipped with a heating plate. The heart was filmed for 20 seconds (at 30 fps). All embryos were assessed as dead or alive and up to eight embryos were used for culture. The time taken from removal from dam to examination of heart rate was less than 25 minutes.

2.2.2. Heart rate analysis

By using software from the University of Uppsala, 15 sec of the videos of the embryonic heart were analyzed and the heart rate and rhythm were obtained (Khan et al., 2008). The software measures the heart rate by measuring changes in dark-light intensity in two different areas manually identified as the atria and the ventricles. The light-dark intensity changes of the regions were recorded as a plot with time on the x-axis and heart activity on the y-axis. The forelimb bud frequently obscured the heart in GD14 embryos. In these cases only the ventricular rate was measured.

2.3. Effect of prenatal chronic low dose dofetilide on embryonic birth weight

A preliminary study identified that a daily oral dose of 2.5 mg/kg dofetilide (Pfizer, Central Research, Sandwich, UK) from GD 11-14 resulted in a moderate decrease in litter size. In the follow-up study, 15 females were mated and examined as described above. Once a day at noon on GD 11-14, 2.5 mg/kg dofetilide was administered orally by gavage to 11 of the rats under isoflurane anesthesia. An equivalent volume of water was administered to 4 controls.
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At birth, the litters were reduced to a maximum of eight pups, maximizing the number of male pups. The pups were weighed every second day until weaning and then weekly.

2.4 Effect of prenatal chronic low dose dofetilide on postnatal cardiovascular function

2.4.1 Telemetry implantation

When the first pups reached a weight over 300 g at approximately 8-9 weeks, radio-telemetry blood pressure transmitters (PAC-40, Data Sciences International, St. Paul, MN, U.S.A.) were implanted surgically into the abdominal aorta. The implantation of the transmitters was performed under anesthesia induced by inhalation of 5% isoflurane (IsoFlo®, Abbott Laboratories Inc). The anesthesia was maintained by administering a mix of ketamine (60 mg/kg) and medetomidine (250 µg/kg), 80% of the mixture was administered intraperitoneal and 20% intramuscular. The anesthesia was deemed adequate when the animal did not exhibit a withdrawal reflex to nociceptive stimulation (a pinch of the hind paw).

Once anesthetised, the rat was placed in a supine position and the abdomen shaved. The skin was sterilized with Betadine® and an incision was made through the abdominal skin and along the linea alba (abdominal muscles). The intestines were temporarily moved from the abdominal cavity to expose the aorta and covered in saline-soaked gauze which was kept moist throughout the procedure.

All visible vessels branching from the aorta were occluded with a suture tie. The aorta was clamped proximally and distally with microvascular clamps to occlude blood flow. It was then pierced close to the iliac bifurcation with a 21 gauge needle bent 90 degrees. The tip of the pressure sensing probe was inserted and advanced approximately one centimeter into the aorta. The site was sealed using n-butyl cyanoacrylate tissue adhesive (3M Vetbond ™) and a cellulose patch to promote hemostasis and support fibrin growth. The intestines were carefully repositioned inside the abdominal cavity and the telemetry device was sutured to the
inner surface of the abdominal muscles. Prophylactic procaine penicillin (0.05 ml) was then administered intraperitoneally to prevent infection. The abdominal muscles were sutured back together and the overlying skin was stapled together.

2.4.2. Post-operative care

The rat was administered a subcutaneous injection of 5% glucose (5 ml) to reduce the risk of post-operative dehydration. Carprofen (4 mg/kg) was administered subcutaneously to reduce inflammation and provide analgesia. The alpha-2-agonist atipamezole (1 mg/kg) was administered via intraperitoneal injection to reverse the effects of medetomidine. The rat was then returned to its home cage under a heat lamp and monitored closely for 24 hours. For the following 7 days, rats were monitored and weighed. If any signs of post-operative stress were observed, such as aggression, dehydration, weight loss greater than 15%, or evidence of pain or infection, additional analgesics or antibiotics were administered. Rats were also provided with sweetened jelly containing the non-steroid anti-inflammatory Ibuprofen (0.05 mg/kg) once daily during recovery for analgesia. If signs of post-operative stress were observed for longer than 48 hours, the rat was euthanized.

2.4.3. Stress tests

After recovery, the rats were placed on their respective receivers (DSI) in two separate rooms with two cages in each room, one control and one treated rat, to undergo stress tests. As there were only four telemetry devices, the rats underwent stress testing in groups of four. A total of 8 controls and 8 treated rats underwent the stress tests. The psychological stressor used was air jet stress, a moderate stress in which a 500 kPa jet of oxygen or room air from an air gun was directed to the rat’s face at a distance of 5-10 cm. The air jet stress was administered simultaneously to both rats in each room. The protocol comprised 15 minutes of intermittent puffs of air. These puffs were broken into 9 blocks, each block made up of 3 puffs of air, 2 seconds in duration and 10 seconds apart. A 60 second recovery time was allowed between each block. The animals were exposed to the protocol twice per day at 12pm and 2pm for one week.

2.4.4. Telemetry recording

The pressure signal obtained from the transmitter was converted to a radio frequency signal via a connected transducer. Thereafter the signal was detected and recorded by a receiver at a
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sampling rate of 1000 Hz. The receiver was connected to a computer from which blood
pressure waveforms were obtained using acquisition software (Data Sciences International
Inc).

Blood pressure data was recorded on four occasions; before, twice during and after the 7 days
of stress. These were recorded in the following manner:

(i) Baseline blood pressure was monitored for 24h the day before the first stress test.
Data were collected for the first 5 minutes of every hour for 24 hours.

(ii) On the first day of the stress protocol, 5h of continuous recording began. These
were scheduled to record an hour of resting period before the stress protocol
began, throughout the protocol, and two hours after the final block of puffs,
deeded the recovery period.

(iii) On the last day of stress, another 5h of continuous recording was performed. As
before, recording started an hour before the protocol and ended 2 hours after.

(iv) Finally, resting blood pressure after a week of air jet stress was recorded for 24h.
Again, the data were collected for 5 minutes every hour for 24 hours. Recordings
were scheduled immediately after the final 2 hours of continuous recording.

At the end of the last 24 hour block of continuous monitoring, the rats were euthanized by an
injection of 0.6 ml pentobarbital sodium (120 mg/kg, Lethabarb ®) followed by puncture of
the diaphragm. The telemetry device was then carefully removed.

2.4.5. Telemetry data analysis

Using the software Acquisition® (Data Sciences International Inc) the arterial blood pressure
wave form was digitized and saved. The data was exported from the computer connected to
the receivers and imported for analysis. Thereafter the files were processed using Spike2
software (Cambridge Electronic Design Ltd). 2 rats were excluded from the analysis due to
transmitter failure.

The 24 five-minute sections of the blood pressure wave form were grouped into two blocks,
one 12 hour night block (7pm to 7am) and one 12 hour day (7am to 7pm) block in order to
assess diurnal variation.
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2.4.5.1. Cardiovascular parameters

By running a customized script (from CED) on the blood pressure wave form within Spike2, heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), pulse pressure (PP) and pulse interval (PI) were obtained off line from the raw signal. Thereafter the mean values of the cardiovascular parameters were calculated for each rat. PP is the difference between SBP and DBP and PI is the period between two consecutive systolic pulses.

2.4.5.2. Autonomic parameters

Autonomic parameters, such as sympathetic nerve activity are usually measured directly in anaesthetized animals using invasive surgical procedures. However an indirect method was used here that provides an index of autonomic function. In this experiment the four autonomic measurements; heart rate variability (HRV) and systolic BP variability (BPV), spontaneous baroreflex sensitivity (sBRS) and baroreflex effectiveness index (BEI) were calculated with non-invasive measurements using the blood pressure wave form and customized scripts within the Spike 2 software (CED).

Heart rate variability (HRV)

Heart rate variability is a measurement of how the heart rate varies over time and is an important marker of autonomic function. Using a customized script within Spike 2 software, HRV was calculated by spectral analysis. A fast Fourier transformation (512 point) was performed on the heart rate wave form and the power spectrum was separated into three predetermined frequency bands, very low frequency (VLF) (0-0.25 Hz), low (LF) - (0.25-0.75 Hz) and high frequency (HF) (0.75-3.3 Hz). The LF band has been shown to correspond to cardiac sympathetic activity while the high frequency band has been shown to correspond to parasympathetic activity (Malik et al., 1996). Hence, if LF is increased it indicates an increase in sympathetic activity and if HF is increased it indicates an increase in parasympathetic activity.

Blood pressure variability (BPV)

Systolic blood pressure variability is a measure of beat-to-beat fluctuations in SBP. Small variations in blood pressure are common even at rest, and can be greater during activity or stress. The analysis is similar to that of HRV, a fast Fourier transformation is performed on
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the systolic blood pressure wave form and the result is split into three bands with identical frequencies as in HRV; VLF (0-0.25 Hz), LF (0.25-0.75 Hz) and HF (0.75-3.3 Hz). (Waki et al., 2006). The low frequency band has been shown to correspond to cardiac sympathetic activity. Unlike the HF band of HRV, the HF band of BPV is unlikely to provide any information about parasympathetic activity because there is no significant parasympathetic innervation of the blood vessels (Waki et al., 2006, Cerutti et al., 1991).

Prior to statistical analysis, outliers were first removed. For HRV and BPV, this consisted of removal of values that fell outside of the mean ± 2 standard deviations.

Spontaneous baroreflex sensitivity (sBRS) and baroreflex index (BEI)

sBRS is a measurement of how much the heart rate changes for a given change in blood pressure and is one way of describing the function of the baroreflex. If the baroreflex is functioning properly, an increase in blood pressure should generate a decrease in heart rate. In the rat, there is normally a delay of 3-5 beats between a change in SBP and reflex response in heart rate (Oosting et al., 1997).

sBRS was calculated by scanning the blood pressure wave form within the Spike2 data analysis software (CED, Cambridge) using a customized script (algorithm). The script recognizes sequences of beats called pressor ramps in which increases in SBP are followed by progressive lengthening of PI. Similarly, depressor ramps are sequences where decreases in SBP are followed by shortening of PI. For each ramp, each pair of SBP-PI data points are plotted and a regression line is fitted to the data. The slope of the regression line is indicative of the sensitivity of the baroreflex. (Di Rienzo et al., 2001, Waki et al., 2006).

Prior to statistical analysis, a sBRS sequence was accepted if there was a minimum of three beats of consecutive increasing or decreasing SBP (four SBP-PI pairs), the linear regression was positive and the correlation coefficient was greater than 0.8 for each time delay (3, 4 and 5 beats). If these criteria were fulfilled, then the mean sBRG was calculated with data split between occurrences when SBP change was positive (pressor) or negative (depressor).

BEI is a measurement of how often the baroreflex is initiated in response to a change in blood pressure and was calculated as the ratio between the number of SBP ramps that generated a baroreflex response (sBRS sequence) and the total number of SBP ramps observed for a given time period. (Di Rienzo et al., 2001, Bajic et al., 2010).
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2.5. Statistics

All statistical analyses were performed using SPSS software (version 21.0). Independent samples t-tests were used to determine if there were differences between control and treated rats for the following parameters: pup weights from PND 3-59 and embryonic heart rates in vitro 2 and 9 hours after dosing. For the radiotelemetry studies, data was split (day from night) before one-way ANOVA was used to compare cardiovascular (HR, MBP, DBP, SBP) and autonomic (sBRS, BEI, HRv, BPv) parameters of control and dofetilide exposed rats. Paired sample t-tests were used to compare cardiovascular and autonomic parameters from day compared to night and before and after the period of the air puff stimuli.

For each variable, normal distribution was assessed by Shapiro-Wilk's test, and homogeneity of variances was assessed by Levene's test for equality of variances. A p-value less than 0.05 were considered statistically significant. All quantitative data are presented as mean ±SEM.
3. Results

3.1. Fetal heart rates and embryonic growth during pregnancy

A hypothesis for this study was that four daily treatments of pregnant rats on GD 11-14 with dofetilide would induce a period of bradycardia in the embryos on each treatment day. This was investigated by examining the embryonic heart rate in at least one litter 2 hours after dosing on each treatment day (Figure 1). In order to determine the duration of bradycardia the heart rate 9 hours after dosing was examined on GD12-14 (Figure 1).

![Graph showing mean % change in HR.]

**Figure 2.** Effect of dofetilide on heart rate on different gestational days

Two hours after dosing, the average heart rates in the dofetilide exposed pups were ~30-45% slower than the heart rates in the control embryos on each gestation day (Figure 2, 3 and 4). The slow heart rates were also sometimes irregular (Figure 3B). Nine hours after treatment, heart rates had mostly recovered although the heart rate at GD13 was still reduced (Figure 2). After the fourth treatment on GD 14 the litters showed increased embryonic death at both collection times. In the litter examined 2 hours after treatment 22% of embryos were dead and in the 9 hour litter 31% of the embryos were dead. In GD 14 controls 2 and 9 hours after treatment there were no dead embryos.
Figure 3. Traces of the embryonic heart lasting 10 sec. Traces are normalised for each embryo ranging from end-systole (0) to end diastole (1). The trace for the atrium is red and for the ventricle blue. (A) GD13 control 2h after dosing (B) GD13 dofetilide 2h after dosing (C) GD13 control 9h after dosing (D) GD13 dofetilide 9h after dosing.
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Figure 4. (A) Rat embryonic HR in embryos collected 2 or 9h after dosing with water or dofetilide. Note, HR returns to normal on GD 12 and 14. (B) Gross morphology and size of rat embryos GD11 to 14

The embryonic growth was measured using crown-rump length (CRL) which is the length of the embryo from the top of the crown (head) to the bottom of the rump. As seen in Figure 4, the rat embryo grows very rapidly between GD11 and GD14, increasing in crown-rump length from ~4mm to ~13.5 mm. The repeated periods of dofetilide-induced bradycardia were expected to cause growth retardation. While there was no significant difference in CRL between control and treated embryos examined 2 hours after dosing on GD 14 by 9 hours, CRL was significantly reduced (13%) when compared to the control embryos (Figure 5).
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<table>
<thead>
<tr>
<th>Control 2h</th>
<th>Dofetilide 2h</th>
<th>Control 9h</th>
<th>Dofetilide 9h</th>
</tr>
</thead>
</table>

Figure 5. GD14 embryos 2 and 9 hours after fourth day of dosing. Note that the dofetilide exposed embryo is growth retarded 9 hours after the last dose. There is also evidence of blood stasis at the root of the forelimb.

3.2. Offspring body weights

All pups were born on GD 22 or 23. In total 11 rats were treated with dofetilide and there were four control rats. Of the 11 treated rats, only 5 produced a litter. Of the 5 dofetilide-exposed dams, two produced litters of one pup, one of which died at birth and the other survived. The mean litter size of the remaining three litters was significantly smaller than control litters (10.0 ± 4.2 compared with 14.3±0.5). Litter sizes were reduced to a maximum of 8 at 3 days postnatal.

A second hypothesis for this study was that embryos exposed to dofetilide during GD11-14 would have lower birth weights than normal. Since only male rats were used in the adult part of the study only weights of the male offspring are given.

The pups were not weighed until postnatal day 3 to avoid disturbing the dam. At 3 days of age, male dofetilide exposed pups were significantly 9.8% lighter (p=0.024) than pups compared to control litters (Table 1). During the lactation period the dofetilide pups increased in weight faster than the controls. After weaning at ~day 28 the dofetilide rats were significantly lighter than controls, on average 8-9% difference.
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Table 1: Birth weights in dofetilide treated and control offspring. Significant difference between groups at birth and post-weaning to 59 days of age.

<table>
<thead>
<tr>
<th>postnatal day</th>
<th>number of control rats</th>
<th>weight (g)</th>
<th>number of dofetilide rats</th>
<th>weight (g)</th>
<th>% difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>31</td>
<td>9.2±1.1</td>
<td>11</td>
<td>8.3±1.6</td>
<td>-9.8* (p=0.024)</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>12.6±1.6</td>
<td>12</td>
<td>12.3±1.8</td>
<td>-2.2</td>
</tr>
<tr>
<td>7</td>
<td>31</td>
<td>17.0±2.4</td>
<td>12</td>
<td>16.5±2.4</td>
<td>-3.0</td>
</tr>
<tr>
<td>10</td>
<td>31</td>
<td>23.2±2.6</td>
<td>12</td>
<td>23.5±2.9</td>
<td>+1.3</td>
</tr>
<tr>
<td>17</td>
<td>31</td>
<td>41.7±2.6</td>
<td>12</td>
<td>41.4±4.8</td>
<td>-0.5</td>
</tr>
<tr>
<td>24</td>
<td>31</td>
<td>71.3±3.7</td>
<td>12</td>
<td>69.2±5.6</td>
<td>-3.0</td>
</tr>
<tr>
<td>31</td>
<td>31</td>
<td>118.8±7.1</td>
<td>12</td>
<td>108.5±8.5</td>
<td>-8.7* (p&lt;0.001)</td>
</tr>
<tr>
<td>38</td>
<td>31</td>
<td>183.2±12.2</td>
<td>12</td>
<td>168.3±12.3</td>
<td>-8.1* (p=0.002)</td>
</tr>
<tr>
<td>45</td>
<td>31</td>
<td>246.5±16.5</td>
<td>12</td>
<td>227.3±15.0</td>
<td>-7.8* (p=0.003)</td>
</tr>
<tr>
<td>52</td>
<td>31</td>
<td>311.6±22.3</td>
<td>12</td>
<td>287.9±19.1</td>
<td>-7.6* (p=0.007)</td>
</tr>
<tr>
<td>59</td>
<td>31</td>
<td>373.7±23.6</td>
<td>12</td>
<td>336.8±27.0</td>
<td>-9.9* (p&lt;0.001)</td>
</tr>
</tbody>
</table>

*p<0.05, significant difference between controls and treated

Figure 6. (A) fused digits (4-5) on forelimb (B) Kinked tail.

Of the 12 dofetilide treated male offspring, 2 showed evidence of malformation, one had a kinked tail and another had fused digits on the forelimb (Figure 6). This rat died during surgery. All control offspring appeared normal.

3.3. Cardiovascular parameters at rest

Control and dofetilide treated offspring were implanted with transmitters when they reached a weight over 300 g at approximately 8-9 weeks. After a period of 1 week recovery the animals were placed in the recording room. There were 8 control and 8 treated rats. Two of the control rats were subsequently removed from the analysis due to methodological problems and not replaced.

The telemetry recording generated a blood pressure wave form from which the cardiovascular parameters were calculated. The cardiovascular parameters that were examined were heart
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...rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), pulse interval (PI) and pulse pressure (PP). PP is the difference between SBP and DBP and PI is the period between two consecutive systolic pulses.

3.3.1. Differences between day and night

Because some cardiovascular parameters are known to vary diurnally the data was split into day and night to examine this difference. The day was defined as the 12 hours between 7 am until 7 pm and the night was defined as 7 pm until 7 am. The results showed that there was significant diurnal variation in HR and PI in both controls and treated rats. HR was >10% higher during the night while PI was lower during the night in both control (Table 2, Figure 7) and dofetilide exposed rats (Table 3, Figure 7). PP in controls was significantly lower during the night (Table 2, Figure 7).

| Table 2. Effect of diurnal variation on cardiovascular parameters in control rats |
|---------------------|--------|--------|--------|--------|
|                     | Day    | Night  | %      | p-values |
| HR (bpm)            | 346.1±9.4 | 384.2±8.8 | +11.0% | 0.014   |
| SBP (mm Hg)         | 100.6±1.8 | 100.2±2.1 | -0.4%  | 0.892   |
| DBP (mm Hg)         | 65.7±2.4  | 67.5±1.8  | +2.7%  | 0.562   |
| MBP (mm Hg)         | 80.9±2.0  | 81.9±1.8  | +1.2%  | 0.719   |
| PI (msec)           | 177.3±4.91 | 160.1±3.5 | -9.7%  | 0.017   |
| PP (mm Hg)          | 35.1±1.4  | 32.8±1.7  | -2.3%  | 0.312   |
Does prenatal hypoxia lead to permanent cardiovascular change in the offspring?

Table 3. Effect of diurnal variation on cardiovascular parameters in dofetilide exposed rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day</th>
<th>Night</th>
<th>%</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>331.3±9.9</td>
<td>373.0±8.7</td>
<td>+12.6</td>
<td>0.007</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>109.3±3.2</td>
<td>111.2±3.0</td>
<td>+1.8</td>
<td>0.664</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>76.5±2.5</td>
<td>79.0±2.4</td>
<td>+3.3</td>
<td>0.478</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>89.3±2.4</td>
<td>91.8±2.3</td>
<td>+2.8</td>
<td>0.470</td>
</tr>
<tr>
<td>PI (msec)</td>
<td>185.2±5.4</td>
<td>165.2±3.7</td>
<td>-10.8</td>
<td>0.009</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>35.4±2.6</td>
<td>34.7±2.5</td>
<td>-1.9</td>
<td>0.855</td>
</tr>
</tbody>
</table>

3.3.2. Differences between controls and dofetilide exposed rats

The cardiac parameters recorded during the day (Table 4, Figure 7) and during the night (Table 5) from controls and dofetilide treated rats were compared. MBP and DBP were significantly higher in the dofetilide exposed rats compared to controls during the day (Table 4, Figure 7)

Table 4. Differences in cardiovascular parameters between controls and dofetilide exposed rats during the day.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Dofetilide</th>
<th>%</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>346.1±9.4</td>
<td>331.3±9.9</td>
<td>-4.3</td>
<td>0.313</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>100.6±1.8</td>
<td>109.3±3.169</td>
<td>+8.6</td>
<td>0.052</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>65.7±2.4</td>
<td>76.5±2.5</td>
<td>+16.5</td>
<td>0.010</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>80.9±2.0</td>
<td>89.3±2.4</td>
<td>+10.4</td>
<td>0.026</td>
</tr>
<tr>
<td>PI</td>
<td>177.3±4.9</td>
<td>185.2±5.4</td>
<td>+4.5</td>
<td>0.317</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>35.1±1.4</td>
<td>35.4±2.6</td>
<td>+0.9</td>
<td>0.922</td>
</tr>
</tbody>
</table>
Does prenatal hypoxia lead to permanent cardiovascular change in the offspring?

At night SBP, DBP and MBP were all significantly increased in the dofetilide treated rats (Table 5, Figure 7). Differences in HR, PI and PP were not significant.

Table 5. Differences in cardiovascular parameters between controls and dofetilide exposed rats during the night.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Dofetilide</th>
<th>%</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>384.2±8.8</td>
<td>373.0±8.7</td>
<td>-2.9</td>
<td>0.392</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>100.2±2.1</td>
<td>111.2±3.0</td>
<td>+10.8</td>
<td>0.017</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>67.5±1.8</td>
<td>79.0±2.4</td>
<td>+17.1</td>
<td>0.004</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>81.9±1.8</td>
<td>91.8±2.3</td>
<td>+12.1</td>
<td>0.007</td>
</tr>
<tr>
<td>PI (msec)</td>
<td>160.1±3.5</td>
<td>165.2±3.7</td>
<td>+3.2</td>
<td>0.348</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>32.8±1.7</td>
<td>34.7±2.5</td>
<td>+6.0</td>
<td>0.53</td>
</tr>
</tbody>
</table>
Does prenatal hypoxia lead to permanent cardiovascular change in the offspring?

Figure 7. Cardiovascular parameters in controls and dofetilide exposed rats during 24 hour before stress. (A) Significant diurnal difference in HR in both controls and dofetilide exposed. (B) Significant difference in MBP between control and dofetilide during night and day. (C) Significant diurnal difference in PI in both controls and dofetilide exposed. (D) Significant difference in SBP between controls and dofetilide exposed during the night. (E) No significant differences in PP. (F) Significant difference between control and dofetilide exposed during both day and night.
Does prenatal hypoxia lead to permanent cardiovascular change in the offspring?

3.4. Cardiovascular parameters after one week of air-jet stress

For a period of 7 days the rats were subjected to air jet stress twice a day. After completion of the week of stress, the baseline blood pressure was again monitored for 24 hours and data were collected for the first 5 minutes of every hour.

3.4.1. Differences between day and night

The diurnal variability was examined by splitting the data into day and night in the same way as previously described. Heart rate and PI again displayed diurnal variation in control rats (Table 6, Figure 8) and dofetilide treated rats (Table 7, Figure 8).

Table 6. Effect of diurnal variation on cardiovascular parameters in control rats

<table>
<thead>
<tr>
<th></th>
<th>Day</th>
<th>Night</th>
<th>%</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>332.1±7.2</td>
<td>384.5±5.6</td>
<td>+15.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>100.9±2.7</td>
<td>101.8±2.3</td>
<td>+0.9</td>
<td>0.794</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>68.8±3.5</td>
<td>72.0±3.3</td>
<td>+4.7</td>
<td>0.518</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>82.5±3.1</td>
<td>84.9±2.7</td>
<td>+2.9</td>
<td>0.573</td>
</tr>
<tr>
<td>PI (msec)</td>
<td>185.6±3.6</td>
<td>159.3±2.6</td>
<td>-14.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>32.1±1.7</td>
<td>29.9±2.1</td>
<td>+7.1</td>
<td>0.414</td>
</tr>
</tbody>
</table>

Table 7. Effect of diurnal variation on cardiovascular parameters in dofetilide exposed rats

<table>
<thead>
<tr>
<th></th>
<th>Day</th>
<th>Night</th>
<th>%</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>313.8±10.1</td>
<td>366.5±10.4</td>
<td>+16.8</td>
<td>0.003</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>112.1±1.6</td>
<td>115.1±2.0</td>
<td>+2.7</td>
<td>0.252</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>76.6±2.2</td>
<td>79.8±2.6</td>
<td>+4.2</td>
<td>0.361</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>91.9±1.7</td>
<td>95.2±2.2</td>
<td>+3.6</td>
<td>0.267</td>
</tr>
<tr>
<td>PI (msec)</td>
<td>195.7±5.9</td>
<td>167.5±4.8</td>
<td>-14.4</td>
<td>0.002</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>35.3±1.8</td>
<td>35.3±1.8</td>
<td>-0.5</td>
<td>0.950</td>
</tr>
</tbody>
</table>
3.4.2. Differences between controls and dofetilide exposed rats

The cardiac parameters recorded during the day (Table 8, Figure 8) and the night (Table 9, Figure 8) from controls and dofetilide treated rats were compared. The results show that SBP and MBP were all significantly higher in treated rats compared to controls during the day (Table 8, Figure 8) while SBP and MBP were significantly higher during the night (Table 9, Figure 8). There was no significant difference in HR, PI or PP between control and treated rats during day or night (Table 8 and 9, Figure 7).

Table 8. Differences in cardiovascular parameters between controls and dofetilide exposed rats during the day.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dofetilide</th>
<th>%</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>332.1±7.2</td>
<td>313.8±10.1</td>
<td>-5.5</td>
<td>0.193</td>
</tr>
<tr>
<td>SBP(mm Hg)</td>
<td>100.9±2.7</td>
<td>112.1±1.6</td>
<td>+11.1</td>
<td>0.003</td>
</tr>
<tr>
<td>DBP(mm Hg)</td>
<td>68.8±3.5</td>
<td>76.6±2.2</td>
<td>+11.4</td>
<td>0.070</td>
</tr>
<tr>
<td>MBP(mm Hg)</td>
<td>82.5±3.1</td>
<td>91.9±1.7</td>
<td>+11.3</td>
<td>0.016</td>
</tr>
<tr>
<td>PI (msec)</td>
<td>185.6±3.6</td>
<td>195.7±5.9</td>
<td>+5.4</td>
<td>0.211</td>
</tr>
<tr>
<td>PP(mm Hg)</td>
<td>32.1±1.7</td>
<td>35.5±1.8</td>
<td>+10.4</td>
<td>0.215</td>
</tr>
</tbody>
</table>

Table 9. Differences in cardiovascular parameters between controls and dofetilide exposed rats during the night.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dofetilide</th>
<th>%</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>384.5±5.6</td>
<td>366.46±10.409</td>
<td>-4.7</td>
<td>0.193</td>
</tr>
<tr>
<td>SBP(mm Hg)</td>
<td>101.8±2.3</td>
<td>115.1±2.0</td>
<td>+13.0</td>
<td>0.001</td>
</tr>
<tr>
<td>DBP(mm Hg)</td>
<td>72.0±3.3</td>
<td>79.8±2.6</td>
<td>+10.8</td>
<td>0.084</td>
</tr>
<tr>
<td>MBP(mm Hg)</td>
<td>84.9±2.7</td>
<td>95.2±2.2</td>
<td>+12.1</td>
<td>0.012</td>
</tr>
<tr>
<td>PI(msec)</td>
<td>159.3±2.6</td>
<td>167.5±4.8</td>
<td>+5.2</td>
<td>0.194</td>
</tr>
<tr>
<td>PP(mm Hg)</td>
<td>29.9±2.1</td>
<td>35.3±1.8</td>
<td>+18.3</td>
<td>0.071</td>
</tr>
</tbody>
</table>
Does prenatal hypoxia lead to permanent cardiovascular change in the offspring?

3.4.3. Differences between pre-stress and post-stress

Paired sample t-tests were used to compare variables from the first 24h recordings with those recorded after one week of air jet stress. The pre- and post-stress comparison for day-time cardiovascular parameters in controls showed significant decrease in PP after stress (Table 10, Figure 8). The pre- and post-stress comparison for night-time cardiovascular parameters in controls showed significant increases in SBP, DBP and MBP after stress (Table 11, Figure 8).

Table 10. Differences in cardiovascular parameters post-stress compared to pre-stress in controls during the day.

<table>
<thead>
<tr>
<th></th>
<th>Pre-stress</th>
<th>Post-stress</th>
<th>%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>346.1±9.4</td>
<td>332.1±7.2</td>
<td>-4.0</td>
<td>0.188</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>100.6±1.8</td>
<td>100.9±2.7</td>
<td>+0.3</td>
<td>0.794</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>65.7±2.4</td>
<td>68.8±3.5</td>
<td>+4.7</td>
<td>0.068</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>80.9±2.0</td>
<td>82.5±3.1</td>
<td>+2.1</td>
<td>0.223</td>
</tr>
<tr>
<td>PI (msec)</td>
<td>177.3±4.9</td>
<td>185.6±3.6</td>
<td>+4.7</td>
<td>0.121</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>35.1±1.4</td>
<td>32.1±1.7</td>
<td>-8.4</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 11. Differences in cardiovascular parameters post-stress compared to pre-stress in controls during the night.

<table>
<thead>
<tr>
<th></th>
<th>Pre-stress</th>
<th>Post-stress</th>
<th>%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>384.2±8.8</td>
<td>384.5±5.6</td>
<td>+0.1</td>
<td>0.975</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>100.2±2.1</td>
<td>101.8±2.3</td>
<td>+1.6</td>
<td>0.039</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>79.0±2.4</td>
<td>72.0±3.3</td>
<td>+6.7</td>
<td>0.036</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>81.9±1.8</td>
<td>84.9±2.7</td>
<td>+3.7</td>
<td>0.038</td>
</tr>
<tr>
<td>PI (msec)</td>
<td>160.1±3.5</td>
<td>159.3±2.6</td>
<td>-0.5</td>
<td>0.758</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>32.8±1.7</td>
<td>29.9±2.1</td>
<td>-8.9</td>
<td>0.064</td>
</tr>
</tbody>
</table>
Does prenatal hypoxia lead to permanent cardiovascular change in the offspring?

The pre- and post-stress comparison for day-time cardiovascular parameters in dofetilide treated rats showed significant increase in PI as well as a significant decrease in HR (Table 12, Figure 8). The pre- and post-stress comparison for night-time cardiovascular parameters in dofetilide treated rats showed significant increases in SBP and MBP (Table 13, Figure 8).

Table 12. Differences in cardiovascular parameters post-stress compared to pre-stress in dofetilide exposed rats during the day.

<table>
<thead>
<tr>
<th></th>
<th>At rest</th>
<th>Post stress</th>
<th>%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>331.3±9.9</td>
<td>313.8±10.1</td>
<td>-5.3</td>
<td>0.006</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>109.3±3.2</td>
<td>112.1±1.6</td>
<td>+2.6</td>
<td>0.201</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>76.5±2.5</td>
<td>76.6±2.2</td>
<td>+0.1</td>
<td>0.971</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>89.3±2.4</td>
<td>91.9±1.7</td>
<td>+2.9</td>
<td>0.111</td>
</tr>
<tr>
<td>PI (msec)</td>
<td>185.2±5.4</td>
<td>195.7±5.9</td>
<td>+5.7</td>
<td>0.003</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>35.4±2.6</td>
<td>35.5±1.8</td>
<td>+0.3</td>
<td>0.946</td>
</tr>
</tbody>
</table>

Table 13. Differences in cardiovascular parameters post-stress compared to Pre-stress in dofetilide exposed rats during the night.

<table>
<thead>
<tr>
<th></th>
<th>Pre-stress</th>
<th>Post stress</th>
<th>%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>373.0±8.7</td>
<td>366.5±10.4</td>
<td>-1.8</td>
<td>0.152</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>111.2±3.0</td>
<td>115.1±2.0</td>
<td>+3.5</td>
<td>0.050</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>79.0±2.4</td>
<td>79.8±2.6</td>
<td>+1.0</td>
<td>0.801</td>
</tr>
<tr>
<td>MBP (mm Hg)</td>
<td>91.8±2.3</td>
<td>95.2±2.2</td>
<td>+3.7</td>
<td>0.031</td>
</tr>
<tr>
<td>PI (msec)</td>
<td>165.2±3.7</td>
<td>167.5±4.8</td>
<td>+1.4</td>
<td>0.374</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>34.7±2.5</td>
<td>35.3±1.8</td>
<td>+1.7</td>
<td>0.623</td>
</tr>
</tbody>
</table>
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Figure 8. Cardiovascular parameters in controls and dofetilide treated rats during 24 hours after stress. (A) Significant diurnal difference in control and treated. Significant difference (SD) during the day in treated post stress. (B) SD between control and treated during night and day. SD after stress at night in both controls and treated. (C) Significant diurnal difference between control and dofetilide treated. SD during the day in dofetilide treated post stress (D) SD between control and treated during night and day. (D),(E),(F) SD in SBP and DBP after stress at night and in PP in day after stress in controls.
3.5. Autonomic parameters at rest and after stress

In this analysis the four autonomic measurements; heart rate variability (HRV) and systolic blood pressure variability (BPV), spontaneous baroreflex sensitivity (sBRS) and baroreflex effectiveness index (BEI) were calculated from the recorded blood pressure signal within the Spike 2 software (CED).

3.5.1. Assessment of sympathetic and parasympathetic activity

3.5.1.1. Heart rate variability (HRV)

Heart rate variability is a measurement of how the heart rate varies over time. Fluctuations in the heart rate wave form were separated into three pre-determined frequency bands, very low frequency (VLF) (0-0.25 Hz), low (LF) (0.25-0.75 Hz) and high frequency (HF) (0.75-3.3 Hz). The low frequency band has been shown to correspond to cardiac sympathetic activity while the high frequency band has been shown to correspond to parasympathetic activity (Malik et al., 1996).

Although there was very little evidence of diurnal variation, to maintain consistency, data was again split. In dofetilide treated rats, the analysis of HRV before stress showed diurnal differences in LF \((p=0.045)\) and HF \((p=0.01)\) (Table 14). However this finding was not observed after stress. There were no significant diurnal differences in control rats before or after stress.

There were no significant differences between control and dofetilide rats, in LF or HF frequency ranges during the day or at night before or after stress (Table 14 and 15). In addition there was no difference in HRV during the day or at night when measurements taken before and after stress were compared (Table 14 and 15).
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Table 14. HRV pre-stress (baseline)

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.21±0.07</td>
<td>0.67±0.21</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>0.17±0.04*</td>
<td>0.50±0.07*</td>
</tr>
<tr>
<td>Night</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.27±0.04</td>
<td>0.84±0.16</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>0.33±0.07</td>
<td>0.89±0.13</td>
</tr>
</tbody>
</table>

*p<0.05, day v night. No other comparisons were significant at p<0.05.

Table 15. HRV post-stress

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.52±0.36</td>
<td>0.85±0.43</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>0.21±0.05</td>
<td>0.87±0.26</td>
</tr>
<tr>
<td>Night</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.46±0.19</td>
<td>1.12±0.39</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>0.26±0.105</td>
<td>0.83±0.08</td>
</tr>
</tbody>
</table>

No comparisons were significant at p<0.05.

3.5.1.2. Blood Pressure variability (BPV)

Blood pressure variability is a measure of beat-to-beat fluctuations in SBP. The analysis is similar to the one of HRV, the blood pressure wave form was split into three bands with identical frequencies as in HRV: VLF (0-0.25 Hz), LF(0.25-0.75 Hz) and HF (0.75-3.3 Hz) (Waki et al., 2006).

The LF band has been shown to correspond to cardiac sympathetic activity. Unlike the HF band of HRV, the HF band of BPV is unlikely to provide any information about
parasympathetic activity because there is no significant parasympathetic innervation of the blood vessels (Cerutti et al., 1991, Waki et al., 2006).

There was a significant diurnal difference in LF-BPV in treated rats before stress. After stress, LF-BPV displayed significant diurnal differences in both controls and treated rats (Table 16). There were no significant differences in BPV between control and treated rats before or after stress (Table 16).

When BP data was compared before and after stress it was observed that control rats exhibited a significant decrease in LF after stress during the day but not during the night (Table 17). This was not observed in dofetilide treated rats.

### Table 16. BPV before and after stress

<table>
<thead>
<tr>
<th></th>
<th>LF pre-stress</th>
<th>LF post-stress</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.04±0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.03±0.002&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>0.03±0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03±0.003&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Night</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.05±0.006</td>
<td>0.05±0.003</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>0.06±0.005</td>
<td>0.05±0.004</td>
</tr>
</tbody>
</table>

<sup>a</sup><sub>p<0.05, day v night</sub>  <sup>b</sup><sub>p<0.05, first v last 24h</sub>

### 3.5.1.3 Spontaneous baroreflex sensitivity (sBRS) and Baroreflex effectiveness index (BEI)

sBRS is a measurement of how much the heart rate changes for a given change in blood pressure and is a way of describing the strength of the baroreflex. BEI is a measurement of how often the baroreflex is initiated in response to a change in blood pressure.

In the present study sBRS was calculated in controls and treated rats from the 24 hours at rest as well as from the 24 hours post stress. The data was divided into day and night to examine diurnal variability. As in previous calculations, the day was defined as 7 am to 7 pm, and night was defined as 7 pm to 7 am.
The total sBRS and BEI did not show evidence of diurnal variation in control or treated rats before stress (Table 17) or after stress (Table 18).

The data was also divided into pressor sBRS which is the sBRS when blood pressure change is positive (i.e. blood pressure is rising) and depressor sBRS which is the sBRS when the blood pressure change is negative (i.e. when blood pressure is falling).

**Diurnal change:** Dofetilide rats showed no evidence of diurnal variation pre-stress but greater pressor signal during the day post-stress (*p*=0.046) (Table 18).

**Treatment change:** There was no difference between control and dofetilide rats at any time point for any variable. The results suggest that there is no difference in baroreflex function between control and dofetilide rats.

**Post-stress changes:** Only control rats showed significant changes in sBRS when measurement of depressor signal at night was compared before and after stress with higher measurements observed after stress (*p*=0.034) (Table 17).

**Pressor compared to depressor:** Dofetilide treated rats showed significant differences between pressor and depressor signal pre-stress during the day (*p*=0.014) while control rats displayed differences during the night pre-stress (*p*=0.023).

Table 17. sBRS and BEI pre-stress (baseline).

<table>
<thead>
<tr>
<th>Day</th>
<th>Depressor sBRS (ms.mmHg-1)</th>
<th>Pressor sBRS (ms.mmHg-1)</th>
<th>Total sBRS (ms.mmHg-1)</th>
<th>BEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.4±0.11</td>
<td>1.4±0.07</td>
<td>1.4±0.08</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>1.4±0.17(b)</td>
<td>1.6±0.17</td>
<td>1.5±0.17</td>
<td>0.19±0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Night</th>
<th>Depressor sBRS (ms.mmHg-1)</th>
<th>Pressor sBRS (ms.mmHg-1)</th>
<th>Total sBRS (ms.mmHg-1)</th>
<th>BEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.2±0.09(a)</td>
<td>1.3±0.13</td>
<td>1.3±0.11</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>1.3±0.14</td>
<td>1.4±0.13</td>
<td>1.4±0.13</td>
<td>0.18±0.01</td>
</tr>
</tbody>
</table>

\(a\) *p* <0.05 first 24h v last 24h \(b\) *p* <0.05, depressor v pressor. No other comparisons were significant at *p*<0.05
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Table 18. sBRS and BEI post-stress.

<table>
<thead>
<tr>
<th>Day</th>
<th>Depressor sBRS (ms.mmHg⁻¹)</th>
<th>Pressor sBRS (ms.mmHg⁻¹)</th>
<th>Total sBRS (ms.mmHg⁻¹)</th>
<th>BEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.4±0.12</td>
<td>1.5±0.11</td>
<td>1.5±0.11</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>1.5±0.13</td>
<td>1.8±0.19 *</td>
<td>1.7±0.14</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Night</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.3±0.11</td>
<td>1.4±0.08</td>
<td>1.4±0.09</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>Dofetilide</td>
<td>1.3±0.10</td>
<td>1.4±0.12</td>
<td>1.3±0.10</td>
<td>0.18±0.01</td>
</tr>
</tbody>
</table>

*p<0.05 <0.05, day v night. No other comparisons were significant at p<0.05.
4. Discussion

4.1 Fetal heart rates during pregnancy

The first aim of this study was to confirm the effect of dofetilide on embryonic heart rate after exposure on GD 11-14. The embryo culture results confirmed that the dofetilide treatment caused bradycardia. Previous studies have clearly shown that dofetilide-induced bradycardia is associated with hypoxia in the embryo. For example, a recent study showed that a single but higher oral dose of dofetilide on GD13 induced embryonic bradycardia in vivo when observed using ultrasound (Ritchie et al., 2013). This observation was confirmed by in vitro studies (Ritchie et al., 2013). Follow up work showed that the period of bradycardia persisted for at least 6 hours and caused the embryos to become hypoxic for at least 4 hours (Ritchie et al., 2013). Hence, the available data suggests that the rat embryos in the present study experienced a period of hypoxia on each day of treatment.

Duration of bradycardia is also an important variable. In the current study, dofetilide induced bradycardia within two hours after dosing. Nine hours after dosing the heart rates were close to normal. This is consistent with earlier work with a higher dose (5 mg/kg) of dofetilide where it was shown that heart rates remained depressed 4 and 6 hours after dosing but had returned to normal values by 14 hours (Ritchie et al., 2013).

4.2 Offspring body weights and litter size

In the present study, less than half of the treated rats produced a litter and the mean litter size was significantly smaller than control litters. At 3 days of age, dofetilide exposed litters were close to 10% lighter than control litters. The increased embryonic death and fetal growth retardation are consistent with a severe prenatal insult.

During the lactation period the dofetilide pups increased in weight faster than the controls (8.6 fold compared to 7.9 fold). This was possibly related to the smaller litter sizes of the dofetilide pups. Pup weight has been observed to be inversely proportional to litter size, possibly because a small litter size results in less competition for milk (Chahoud and Paumgartten, 2009). After weaning ~day 28 the dofetilide rats were again significantly lighter than controls and stayed that way throughout the study.
Chronic hypoxia during pregnancy is a common insult to fetal development and has been the subject of much study. Models used include, placental disruption (Chen and Zhang, 2011) and placing pregnant animals in hypoxic environments (Ream et al., 2008, Hauton and Ousley, 2009, Coney and Marshall, 2010). For example a study using pregnant mice demonstrated that 8% O₂ for 24h on GD12.5 (equivalent to GD14 in the rat) resulted in 30% fetal death and surviving pups were reduced in weight (Ream et al., 2008).

However, neither of these models examine the specific effect of embryonic hypoxia, the former disrupting nutrient supply and the latter additionally affecting the mother. Maternal appetite is known to be reduced in association with hypoxia (Williams et al., 2005) thus observed decreased pup weight may, in fact, be a consequence of decreased maternal weight gain and not maternal hypoxia (Peyronnet et al., 2002). Since dofetilide does not affect the dam, in the present study it was possible to examine the specific effects of embryonic hypoxia.

Dofetilide has not been tested in this way before, however previous studies have shown similar results. Original developmental toxicity testing by Pfizer dosed rats from GD 6-15 with 2 mg/kg/day (higher doses resulted in complete resorptions). This resulted in reduced litter sizes (24% post-implantation loss) and fetal weight reductions (18% of control) (FDA, 1998). In another study, a single dose of 5 mg/kg dofetilide was administered to pregnant rats on GD 13 (Ritchie et al., 2013) both the litter size and the mean fetal weight were reduced compared to controls.

4.3 Cardiovascular parameters in the offspring

Only male offspring were examined in this part of the study because the estrus cycle of female rats has a strong influence on activity and would be a confounding factor (Takezawa et al., 1994)

4.3.1 Diurnal differences

Rats are nocturnal animals and are much more active at night than during the day. Hence cardiovascular parameters show a marked diurnal variation (Takezawa et al., 1994). In the current study, this diurnal variation was evident as HR increased and PI decreased at night in both control (HR+11%; PI -10.8%) and dofetilide-exposed offspring (HR+12.6%; PI -9.7%).
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This is also observed in humans, but reversed so that heart rate is higher during the day compared to the night since the day is the active period for humans (Biston et al., 1996).

4.3.2 Differences between controls and dofetilide exposed offspring

A comparison of the cardiovascular parameters of the control and dofetilide exposed offspring before stress showed that the dofetilide exposed had significantly higher mean BP in both day (+10.4%) and night periods (+12.1%) compared to controls. This was also evident in for DBP (SBP was also significantly greater during the night). As SBP and DBP increased to a similar extent in dofetilide exposed, PP was similar in control and exposed offspring. Measurements of HR did not show any significant differences between controls and exposed during the day or at night.

This is the first time it has been shown that embryonic hypoxia, without disturbing placental function or maternal wellbeing, can induce hypertension in the offspring at rest. In contrast to the present study, two previous studies did not report an increase in blood pressure following maternal hypoxia (Coney and Marshall, 2010, Peyronnet et al., 2002) which found no difference in these cardiovascular parameters.

There are several possible explanations for these differences. Firstly, a major difference between the present study and previous reports is the manner in which blood pressure was recorded. The previous studies involved chronic indwelling arterial catheters for blood pressure measurement, and one of the studies was conducted under the influence of general anesthesia (Coney and Marshall, 2010). General anesthesia is well known to affect blood pressure regulation (Bencze et al., 2013) while exteriorized catheters have been reported to affect baseline blood pressure values due to effects of stress (Denton et al., 2006). The mean blood pressure in the control groups was considerably higher (20-40 mmHg) in these studies than what is normally reported in the SD rat, suggesting that the experimental conditions evoked a stress response (Denton et al., 2006). It is possible, therefore, that these previous studies failed to observe a difference in blood pressure “at rest” due to the cardiovascular responses to stress associated with the techniques used to measure blood pressure. In contrast, our study utilized radio telemetry, a technique that allows blood pressure measurement in the conscious animal with minimal stress. This “stress-free” measurement of
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blood pressure may have unmasked the pressure difference in the control and dofetilide exposed groups.

Furthermore, both of the previous studies induced hypoxia by placing pregnant dams in a hypoxic chamber at 10% to 12% O$_2$ for 1-2 weeks during gestation (Coney and Marshall, 2010, Peyronnet et al., 2002). In addition to the hypoxic effect on the fetus, this would have a considerable impact on maternal behavior, including feeding, activity levels and stress hormone production (Peyronnet et al., 2002). Moreover, although the level of hypoxia to which the fetuses were exposed in the present study was not determined, it may have been considerably more severe than in previous studies.

The cause of the increased arterial blood pressure in the resting dofetilide offspring in the present study is unknown; however it may be due to the way the embryo responds to hypoxia. One dominant hypothesis is the ‘thrifty phenotype hypothesis’ (Hales and Barker, 2001). This suggests that, under suboptimal conditions, a foetus adapts to guarantee immediate survival by preserving vital functions at the expense of other less immediately critical functions, for example by halting the development of the kidneys in favour of maintaining the function of the heart. This maladaptation may then be observed postnatally as impaired nephrogenesis, vascular dysfunction, microvascular rarefaction or autonomic dysfunction (Nuyt, 2008) any of which could explain the observed hypertension.

4.3.3. Parameters after one week of stress

Daily exposure to acute air jet stress for one week had little effect on cardiovascular parameters. There was a small but significant increase in MBP at night in both dofetilide exposed offspring and controls of about 3.7% compared to before stress, but the differences were similar between groups. There remained no significant difference in HR, PI or PP between control and treated rats, although HR was slightly decreased in the dofetilide exposed offspring compared to before stress. Previous studies have reported that cardiovascular responses to acute stress are exaggerated in offspring programmed with hypoxia (Coney and Marshall, 2010, Peyronnet et al., 2002) and there is evidence that long-term exposure to psychological stress may lead to chronic hypertension at rest (Xiao et al., 2013). This leads to the hypothesis that a long-term exposure to stress may produce a larger increase in blood pressure or HR in the dofetilide exposed offspring. It is possible that the
intensity or duration of our stressor (intermittent air puffs for one week) may not have been large enough to elicit chronic changes in blood pressure. For example Xiao and colleagues (Xiao et al., 2013) exposed rats to twice daily 2 hour-foot shocks for 14 days to elicit a chronic hypertensive response, a much more substantial stressor. Even so, our results do not support the hypothesis that embryonic exposure to dofetilide alters the cardiovascular responses to stress.

4.4. Autonomic function

The main finding in this study was increased BP (both before and after stress) in the dofetilide exposed rats compared to controls. Stress can lead to elevated sympathetic activity in the heart and vasculature causing blood pressure and heart rate to rise (Dampney et al., 2002). It is also believed that programmed hypertension can increase the risk of developing stress-induced hypertension (Tonkiss et al., 1998, O'Regan et al., 2008). This could manifest either by greater increases in sympathetic activity in response to stressor to increases in sympathetic activity to “stressors” that would not cause this in non-programmed subjects. In an attempt to determine the cause of the observed increase in BP, potential autonomic dysfunction was investigated.

4.4.1. HRV and BPV

Analysis of blood pressure variability (BPV) and heart rate variability (HRV) was made as it has been reported that fluctuations in high frequency variability is indicative of parasympathetic activity while low frequency variation corresponds to sympathetic activity (Malik et al., 1996). An increase in sympathetic activity would be one explanation for the increase in BP (Dampney et al., 2002). However the results did not show significant differences between groups. Hence it does not appear that the increased BP observed in the dofetilide-exposed rats was due to changes in sympathetic or parasympathetic activity.

4.4.2. sBRS and BEI

As mentioned previously, there was no significant difference in HR between controls and dofetilide exposed rats despite the elevated BP. One explanation could be that the autonomic control of BP (baroreflex) has been affected in the dofetilide rats. The baroreflex is a homeostatic mechanism that helps maintain the BP. If the BP is elevated, a negative feedback
mechanism causes the heart rate to decrease which in turn causes BP to decrease. Therefore, if the baroreflex was functioning normally, then HR should be lower in the dofetilide treated rats to try to counter the higher BP (Dampney et al., 2002). However, there was no significant difference in the HR of dofetilide rats. This may be explained if the rat has adapted to the new higher blood pressure by “re-setting” of the baroreflex to another “set point”, causing HR to return to normal despite the higher BP. Re-setting of the baroreflex has been observed in humans and rats and can occur rapidly, even when changes in blood pressure are maintained for periods of less than one day (Thrasher, 2005). In addition, the present results showed no difference in how much or how often HR changed in response to changes in BP (sBRS and BEI). The results suggest that there is no change in baroreceptor sensitivity in dofetilide rats.

In summary, the results showed little evidence of changes in autonomic function which could account for the observed hypertension. It is unknown if hypoxia induced changes in renal or vascular formation.

4.5. Limitations of study

4.5.1 Embryo culture

Only a small number of animals (one control and one dofetilide-exposed) were used to examine the severity and duration of the dofetilide-induced bradycardia at each time point. This experiment was done to confirm results from previous studies which showed that all dofetilide-exposed litters displayed a decrease in HR. Therefore increasing the number of litters would not have affected the outcome. To accurately document severity and duration of the bradycardia however would require a large study. For example a sample size of 8 dofetilide and 8 control embryos would allow us to detect a true difference in the mean HR of experimental and control subjects of 15.1 bpm (or ~7.3%) with probability (power) 0.8 (assuming $p=0.05$) at one time point.

There are a number of errors that can lead to additional reduction of HR during the embryo culture, such as lack of capacity of the culturing system to supply enough oxygen to the embryos. Furthermore, the time it takes to manually position the culture bottle under the microscope before video recording can vary. If it takes too long the temperature of the
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culturing fluid may be reduced and since the HR of the embryos is very temperature sensitive this can cause the HR to decrease (NEW, 1978).

4.5.2. Telemetry analysis

For the telemetry analysis, six controls and eight dofetilide exposed pups were used, which is also a relatively small number when the purpose is to detect differences. This means that one or two abnormal results can modify the overall result.

4.5.3. Use of non-invasive determinants of autonomic function

Non-invasive estimates of autonomic and cardiovascular function have the advantage that they can be carried out in a manner that allows normal physiological behavior to be observed. However, the major disadvantage of these techniques is that these measures are all (except BP) indirect estimates and therefore subject to potential errors or over-interpretation. The autonomic parameters, sBRS, BEI, HRV and BPV have all been validated in numerous studies (Waki et al., 2006, Dowell and Houdi, 1997, Malpas, 2002).

4.5.4. Removal of control animals

Data was not analysed during the experiment. It was clear from examination of initial BP data that the data from two rats were unexpected. One rat (control) was hypertensive (SBP was two standard deviations greater than the mean) and was removed from the study. The signal from the second rat was judged to be faulty as the HR was frequently low (nearly 50 bpm below average) suggesting loss of signal between transmitter and receiver. No rats were removed from the dofetilide-treated group.

This is of concern, on the one hand, we wish to compare our dofetilide rats with normal healthy controls, including a hypertensive rat would bias that; if hypertension is part of the normal population of SD rats then it may have been that a hypertensive rat appeared in the dofetilide exposed group but was not noted as we were hoping to detect hypertension.

4.6. Conclusion

Daily periods of bradycardia (and associated hypoxia) in rat embryos during the organogenic period of development caused increased embryonic death and fetal growth retardation. A postnatal consequence was hypertension in the offspring. This alteration in postnatal
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Cardiovascular function was caused by a direct pharmacological effect of the drug dofetilide on the embryos without affecting cardiovascular functions in the pregnant rat.

The study also demonstrated that, following a period of postnatal stress, there was no difference in cardiovascular parameters in dofetilide-exposed or control offspring suggesting that the normal response mechanisms (autonomic control) had not been altered by the dofetilide exposure.

The cause of the hypertension is unknown but analyses suggest that the increased BP observed in dofetilide-exposed rats is not due to changes in sympathetic or parasympathetic activity. While autonomic dysfunction cannot be ruled out, remaining possibilities include impaired nephrogenesis, vascular dysfunction and microvascular rarefaction. This remains to be explored.
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5. References


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