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Gender-specific impairment of *in vitro* sinoatrial node chronotropic responses and of myocardial ischemia tolerance in rats exposed prenatally to betamethasone

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ABSTRACT

Excessive fetal glucocorticoid exposure has been linked to increased susceptibility to hypertension and cardiac diseases in the adult life, a process called fetal programming. The cardiac contribution to the hypertensive phenotype of glucocorticoid-programmed progeny is less known, therefore, we investigated *in vitro* cardiac functional parameters from rats exposed *in utero* to betamethasone.

Pregnant Wistar rats received vehicle (VEH) or betamethasone (BET, 0.1 mg/kg, i.m.) at gestational days 12, 13, 18 and 19. Male and female offspring were killed at post-natal day 30 and the right atrium (RA) was isolated to *in vitro* evaluation of drug-induced chronotropic responses. Additionally, whole hearts were retrograde-perfused in a Langendorff apparatus and infarct size in response to *in vitro* ischemia/reperfusion (I/R) protocol was evaluated.

Male and female progeny from BET-exposed pregnant rats had reduced birth weight, a hallmark of fetal programming. Male BET-progeny had increased basal RA rate, impaired chronotropic responses to noradrenaline and adenosine, and increased myocardial damage to I/R. Though a 12-fold reduction in the negative chronotropic responses to adenosine, the effects of non-metabolisable adenosine receptor agonists 5′-(*N*-ethylcarboxamido)adenosine or 2-Chloro-adenosine were not different between VEH- and BET-exposed male rats. BET-exposed female offspring presented no cardiac dysfunction.

Prenatal BET exposure engenders male-specific impairment of sinoatrial node function and on myocardial ischemia tolerance resulting, at least in part, from an increased adenosine metabolism in the heart. In light of the importance of adenosine in the cardiac physiology our results suggest a link between reduced adenosinergic signaling and the cardiac dysfunctions observed in glucocorticoid-induced fetal programming.

1. Introduction

Adverse intrauterine environment can lead to developmental changes that cause long-term alterations in postnatal life physiology and risk to development of diseases, a process known as fetal programming (Godfrey and Barker, 2001). Different stressors were shown to cause intrauterine programming of disease in experimental models including maternal undernutrition, intrauterine hypoxia and exposure to abuse drugs (Harris and Seckl, 2011; Dasinger and Alexander, 2016). Antenatal glucocorticoid administration, whose increasing evidence suggests to be triggers and mediators of intrauterine programming, is an established obstetric practice employed to enhance fetal lung maturation in pregnancies at risk of preterm delivery (Roberts and Dalziel, 2006). Apart the benefits of antenatal glucocorticoid administration to

Abbreviations: BET, betamethasone; RA, right atrium; I/R, ischemia/reperfusion; NECA, 5′-(*N*-ethylcarboxamido)adenosine; 2-Cl-adenosine, 2-chloro-adenosine; SAN, sinoatrial node; TTC, 2,3,5-Triphenyl-tetrazolium chloride; A1R, adenosine A1 receptor

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neonate survival, excessive glucocorticoid exposure has been associated with decreased birth weight and increased risk of cardiometabolic and brain disorders (Reynolds, 2013).

Changes in offspring postnatal cardiovascular homeostasis are a well defined facet of fetal programming (Barker, 1993; Barker and Fall, 1993). In particular, substantial data from rodent and human studies show a positive relationship between low birth weight and increased risk of hypertension and ischemic heart disease in adult life (Barker et al., 1989; Mercuro et al., 2013; do Carmo Pinho Franco et al., 2003). Mechanistically, altered vasomotor responses of arteries taken from intrauterine programmed animals were linked to the hypertensive phenotype (Brawley et al., 2003; Franco Mdo et al., 2002; Lamireau et al., 2002; Xiao et al., 2009). Less knowledge is available about any cardiac contribution to the hypertensive phenotype of these programmed animals.

Endogenous glucocorticoid surge in the late gestation is important to heart maturation but excessive glucocorticoid exposure may alter the normal trajectory of this process (Rog-Zielinska et al., 2014). To gain further insight on the cardiac effects of excessive prenatal glucocorticoid exposure we investigated the *in vitro* function of sinoatrial node (SAN) and the function and ischemia tolerance of Langendorff-perfused hearts in rats programmed by intrauterine BET administration. Additionally, in light of the reported gender-specific impairment of cardiovascular responses, the cardiac functional parameters from both, male and female rats, were assessed.

2. Materials and methods

2.1. Animals

The experimental procedures were approved by the local Ethics Committee for the Use of Experimental Animals of the University of São Paulo State (protocol number 451-CEEA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). The euthanasia was performed by decapitation and all efforts were made to minimize the number and suffering of animals.

Male (90 days old/300–350 g) and female (90 days old/225–230 g) Wistar rats were obtained from the Multidisciplinary Center for Biological Investigation, State University of Campinas and maintained under controlled conditions (25 °C, 30% air humidity, 12/12-h light/dark cycle) with food and water available *ad libitum*.

The experimental design was performed as published by C.S. Borges et al. (2016) and C.D. Borges et al. (2017). Briefly, each sexually experienced male rat ($n = 10$) was paired with two nulliparous female rats ($n = 20$) during the dark cycle of the photoperiod and allowed to stay together until sperm were detected in the daily vaginal smears as evidence of copulation. The detection of sperm in the vaginal smear of rats in estrus was considered as gestational day 1 (GD1). Pregnant and lactating rats ($n = 15$) were maintained in individual cages and were randomly allocated into two experimental groups: VEH (vehicle; saline solution; $n = 6$) and BET (0.1 mg/kg of betamethasone, Sigma; $n = 9$) treated by intramuscular injection on days 12, 13, 18 and 19 of pregnancy. This injection protocol was based on the maternal corticosteroid therapy previously adopted by Piffer et al. (2009a).

After birth, during 21 days, the pups were maintained with dams and on post-natal day 21 (PND21, weaning) they were housed in separate cages ($n = 3$ pups/litter). For each set of experiments, one male and female rat from each litter was sampled in order to prevent potential variation due to litter effects.

2.2. In vivo mean arterial blood pressure (MAP) and heart rate (HR) recording in conscious unrestrained rats

Due to the limited number of animals available to the realization of all experiments of this study the *in vivo* recording of MAP and HR was done with the male offspring, only. Thirty-day old VEH- and BET-exposed male rats ($n = 5$ for both groups) were anesthetized with 2% isoflurane in 100% O_2 and the left carotid artery was catheterized with a heparin-filled (500 IU/ml) polyethylene catheter (PE50 connected to PE10). The catheter was driven subcutaneously and exteriorized in the dorsum of the rat. Twenty-four hours after the surgery the carotid catheter was connected to a pressure transducer in a MP150 system (Biopac, Santa Barbara, CA, USA) and the MAP and HR were recorded continuously during 15 min in conscious unrestrained rats. All the recordings of hemodynamic parameters were done between 8 am and 12 pm. Due to the known ischemic preconditioning effect of isoflurane (Liu et al., 2015; Lotz et al., 2015) animals included in this experiment were not employed in other assays of this study.

2.3. In vitro RA contraction

Thirty day-old VEH- and BET-exposed male and female rats were killed by decapitation and the heart was excised and immersed in ice-cold Krebs-Henseleit solution of following composition (mM): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 dextrose, pH 7.4. The RA was isolated, washed from remaining blood and attached to isometric force transducers under 1 gram resting tension in 10 ml organ baths filled with Krebs-Henseleit solution (pH 7.4) at 30 °C constantly bubbled with 95%O_2/5%CO_2. Tissues were allowed a 1 hour equilibration period with solution changes every 15 min. After the equilibration period the spontaneous basal RA rate was recorded and cumulative concentration-response curves to noradrenaline, adenosine and acetylcholine were obtained in each tissue at 30 minute interval. In another series of experiments the RA chronotropic responses to 5′-(*N*-ethylcarboxamido)adenosine (NECA) and 2-chloro-adenosine (2-Cl-adenosine), two adenosine receptor agonists resistant to adenosine uptake/metabolism, were investigated in RA from VEH- and BET-exposed male rats.

Data from all rats of the same experimental group were plotted together in GraphPad Prism 5 software (GraphPad, CA, USA) and subjected to non-linear regression analysis using a sigmoidal dose-response curve fitting and the averaged maximal chronotropic responses (E_{max}) expressed as changes of beating rate over the basal atrial rate (Δ atrial rate, in beats per minute (bpm)) and the agonist potencies expressed as pD_2, *i.e.* the negative of logarithm of agonist concentration inducing 50% of maximal response, were evaluated.

2.4. Langendorff-perfused hearts

Male and female VEH- and BET-exposed rats were anesthetized with ketamin + xylazine (70 + 15 mg/kg, respectively). Once anesthesia was achieved heparin (100 IU) were injected intraperitonialy. Then, the heart was excised *via* thoracotomy and immediately immersed in ice-cold Krebs-Henseleit solution of above cited composition except the $CaCl_2$ concentration that was reduced to 1.8 mM, a modification previously used in myocardial ischemia studies to better reflect the physiologically ionized blood Ca^{2+} concentration (Sutherland and Hearse, 2000). The adherent tissues were removed, the aorta was cannulated with a 17 Gauge needle and the heart retrograde-perfused with oxygenated Krebs-Henseleit solution (with 1.8 mM $CaCl_2$) at 37 °C. As altered coronary contraction/relaxation from antenatal BET-exposed lambs was described previously (Segar et al., 2006), the constant flow perfusion mode was employed to allow the measurement of different functional endpoints under the same experimental conditions. As preliminary experiments showed hearts from vehicle or betamethasone-exposed 30 day-old rats averaged approximately 0.8 g, the perfusion was set at constant flow of 8 ml/min in accordance to previously published value of 10 ml/min/g of heart wet weight (Kolar et al., 1990).

To avoid unintended ischemic preconditioning all the procedures from the heart excision to commencing of perfusion were done in < 3 min and hearts out of this time limit were not used. After the perfusion was established, the left auricle was removed and a saline-filled

latex balloon (Harvard apparatus; Chatswood, NSW, Australia) connected to a pressure transducer was inserted into the left ventricle (LV) to record of intraventricular pressure development. The latex balloon was inflated with saline to achieve an end diastolic pressure of 15 mmHg and the heart allowed a 15 minute perfusion with no additional manipulation (equilibration period). The following heart functional parameters were evaluated during the equilibration period: heart rate (HR), left ventricle developed pressure (LVDP), left ventricle end diastolic pressure (LVEDP), maximal rate of contraction (+ dp/dt) and relaxation (− dp/dt).

After the 15 minute equilibration period, the heart was subjected to a 20 minute no-flow normothermic (37 °C) global ischemia followed by a 1 hour reperfusion. The HR and LVDP values were continuously recorded during all the I/R protocol as these two parameters has been extensively shown to reflect the heart functional recovery during the reperfusion period.

2.5. Myocardial infarct size determination

After the end of 1 hour reperfusion period the hearts were cut in four slices and immersed in 1% 2,3,5-Triphenyl-tetrazolium chloride (TTC) solution for 20 min at 37 °C followed by 30 min in formalin. The slices were mounted between a pair of histological glass slides and images of both sides were digitalized. The area of each heart slice and the infarcted areas were quantified by ImageJ 1.50b software (NIH, Bethesda, MD; available at http://imagej.nih.gov/ij/) and the myocardial infarct size was quantified as the ratio of total infarcted area by the sum of total slices area (the area at risk) multiplied by 100. These values represent the percentage of total myocardial area infarcted.

2.6. Statistical analysis

Data are shown as mean ± standard error of mean (S.E.M) from n observations where each n represents one rat from a different litter. Statistical comparisons between VEH- and betamethasone groups were done with unpaired Student's t-test and p values < 0.05 were considered significant.

3. Results

3.1. Intrauterine BET-exposure effects on offspring growth

BET-exposure have no effects on litter sizes (VEH = 9.63 ± 1.01, n = 6; BET = 10.78 ± 0.70, n = 9; p > 0.05 Student's t-test) or litter sex ratio (female/male ratio, VEH = 1.45 ± 0.38, n = 6; BET = 1.80 ± 1.03, n = 9; p > 0.05 Student's t-test). Male and female offspring from BET-exposed showed decreased birth weight (− 15%) as compared to respective VEH-exposed rats. The reduced body weight of male, but not female rats, was maintained until the rats were 30-day old (− 10%; Table 1). No differences in the relative organ weight of heart, kidneys, adrenal, lung or male and female reproductive organs were observed between VEH- and BET-exposed rats (data not shown).

3.2. In vitro RA chronotropic responses

Spontaneous basal RA rate from BET-exposed male rats was 12% higher as compared to VEH-exposed male rats (p < 0.05; Fig. 1A). The potency of noradrenaline-induced positive chronotropic response of RA from BET-exposed male rats was reduced by 2.5-fold whereas the maximum chronotropic response was 18% higher in RA from BET-exposed male rats (Fig. 1B and Table 2).

Adenosine induced concentration-dependent negative chronotropic responses in RA from VEH- and BET-exposed rats. However, albeit adenosine was able to arrest the RA in both groups, the potency of adenosine was 12.5-fold lower in RA from BET-exposed male rats (Fig. 1C and Table 2). The acetylcholine potency or maximal negative chronotropic response in RA from BET-exposed male rats was not different from VEH-exposed rats (Fig. 1D and Table 2).

Neither the spontaneous basal RA rate nor drug-induced chronotropic responses of RA from BET-exposed female rats were different from that of VEH-exposed female rats (Fig. 1E–H and Table 2). Representative recordings of adenosine effects on spontaneous RA contraction rate are provided in Fig. 1I–L.

To further investigate the reduced adenosinergic response of RA from BET-exposed male rats, the negative RA chronotropic responses to NECA and 2-Cl-adenosine, two adenosine agonists resistant to adenosine uptake/metabolism processes, were investigated. As depicted in Fig. 2A and D the negative chronotropic responses to NECA and 2-Cl-adenosine were not different between VEH- and BET-exposed male rats (NECA VEH pD_2:7.35 ± 0.06, E_{max}: − 181.0 ± 4.77 bpm, n = 4 *vs* BET pD_2: 7.20 ± 0.07, E_{max}: − 186.0 ± 5.45 bpm, n = 6; p > 0.05, Student's t-test; 2-Cl-adenosine VEH pD_2:6.03 ± 0.13, E_{max}: − 197.0 ± 14.65 bpm, n = 4 *vs* BET pD_2: 6.05 ± 0.13, E_{max}: − 191.0 ± 13.64 bpm, n = 6; p > 0.05, Student's t-test). In Fig. 2B–C and E–F representative trace-recordings of NECA and 2-Cl-adenosine RA concentration-response curves are shown.

The difference in the potency between the adenosine and its non-metabolisable analogue 2-Cl-adenosine was higher in BET-exposed rats (1.92 ± 0.12, *i.e.* 83-fold, n = 6) as compared to VEH-exposed rats (0.78 ± 0.20, *i.e.* 6-fold, n = 4) further suggesting increased adenosine uptake/metabolism in RA from BET-exposed male rats (p < 0.05, Student's t-test).

3.3. In vivo MAP and HR

MAP and HR from BET-exposed male rats were not different from the values of VEH-exposed male rats (MAP: VEH 80.25 ± 3.61 mmHg, n = 5 *vs* BET 89.03 ± 4.03 mmHg, n = 5, p > 0.05, Student's t-test; HR VEH: 460.6 ± 32.90 bpm, n = 5 *vs* BET: 447.5 ± 22.38 bpm, n = 5; p > 0.05, Student's t-test).

Table 1
Birth weight (BW) and 30-day body weight (FBW) of male and female rats exposed prenatally to VEH or BET.

Male			Female		
	VEH	BET		VEH	BET
BW (g)	7.71 ± 0.39 (n = 6[a])	6.48 ± 0.23* (n = 9[a])	BW (g)	7.23 ± 0.29 (n = 6[a])	6.14 ± 0.12* (n = 9[a])
FBW (g)	109.2 ± 2.47 (n = 14[b])	95.13 ± 2.24* (n = 18[b])	FBW (g)	93.95 ± 4.46 (n = 6[b])	88.55 ± 3.62[ns] (n = 6[b])

Data expressed as mean ± s.e.m.
ns: non-significant (p > 0.05, Student's t-test).
[a] Number of different litters evaluated (weights from all pups of the same sex were averaged by litter)
[b] Number of different rats evaluated.
* p < 0.05 *vs* VEH (Student's t-test)

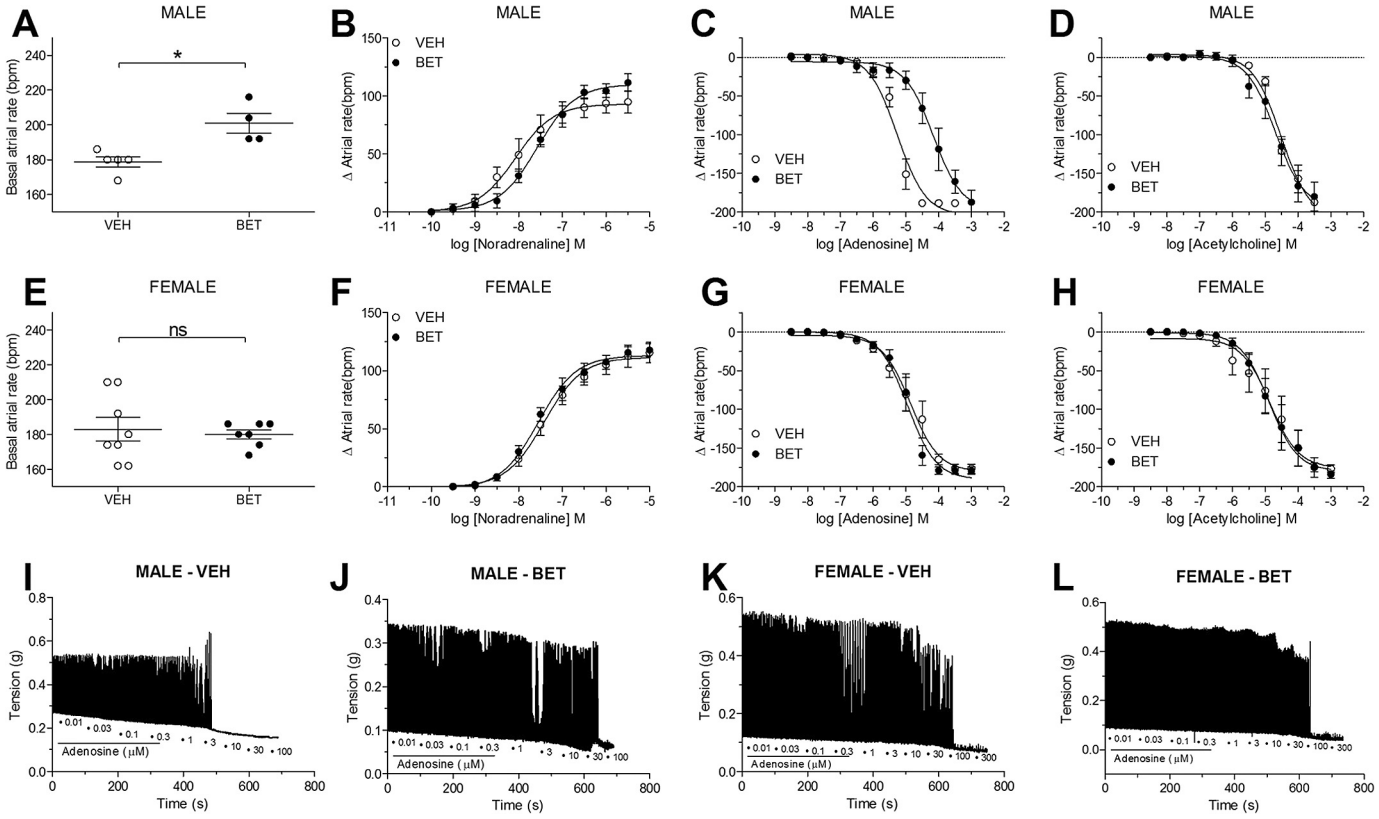

Fig. 1. Antenatal BET-exposure induced stimulus-specific decrease in SAN responses to adenosine and noradrenaline in male rats. Spontaneous RA from VEH- and BET-exposed Male (A) and Female (E) rats. Drug-induced chronotropic responses of RA from Male (B to E) and Female rats (F to H) expressed as beats per minute over basal atrial rate. Representative trace-recordings showing the negative chronotropic responses of RA from male (I and J) and female (K and L) rats exposed to VEH or BET *in utero*. The representative recordings in I–L have tension and time scales arbitrary set to zero. Data in A to H represent mean ± S.E.M. from 5 to 6 rats from different litters. *$p \leq 0.05$ *vs* VEH-exposed rats (Student's *t*-test); ns: non-significant ($p > 0.05$ *vs* VEH-exposed rats, Student's *t*-test).

Table 2
In vitro potency (pD_2) and maximal chronotropic response of RA taken from prenatally VEH- or BET-exposed male and female rats.

	Male			Female		
	VEH	BET	*n*	VEH	BET	*n*
Noradrenaline						
pD_2	8.09 ± 0.17	7.59 ± 0.09*	5	7.42 ± 0.10	7.55 ± 0.08	8
E_{max}	92.96 ± 4.73	110 ± 3.59*	5	111.60 ± 3.79	113.10 ± 3.07	8
Adenosine						
pD_2	5.26 ± 0.07	4.16 ± 0.12*	5	4.86 ± 0.10	4.95 ± 0.08	7
E_{max}	−204.10 ± 6.29	−197.80 ± 12.95	5	−180.40 ± 7.27	−190.60 ± 6.11	7
Acetylcholine						
pD_2	4.51 ± 0.08	4.68 ± 0.18	5	4.86 ± 0.16	4.89 ± 0.13	7
E_{max}	−208.70 ± 10.23	−194.50 ± 12.20	5	−175.70 ± 11.19	−179.80 ± 9.83	7

E_{max} values in bpm.
* $p < 0.05$ *vs* VEH (Student's *t*-test).

3.4. *In vitro* heart function to global I/R

None of the functional parameters of spontaneously beating hearts from BET-exposed male and female rats were different between their respective VEH-exposed controls during the equilibration period (Table 3).

In vitro global I/R impaired the function of hearts from all groups as evaluated by the decrease in the HR and LVDP as compared to pre-ischemic values. Importantly, the functional recovery of hearts from BET-exposed male rats was significantly impaired compared to respective VEH-exposed male rats. The ischemia arrested the hearts from VEH- and BET-exposed rats at same extent but the recovery of HR during the reperfusion period was delayed in hearts from BET-exposed male rats (Fig. 3A). In addition, the LVDP recovery from BET-exposed male rat hearts was lower than the hearts of VEH-exposed male rats from 15 to 60 min of reperfusion (Fig. 3B).

In contrast, the post-ischaemic functional recovery of HR and LVDP from BET-exposed female rats was identical to that of VEH-exposed female rats (Fig. 3D and E). Representative original recordings of intraventricular pressure from Langendorff hearts of VEH- and BET-exposed male and female rats are presented in Fig. 3C and F, respectively.

3.5. Myocardial injury to *in vitro* global I/R

The *in vitro* I/R protocol induced myocardial infarction in hearts from rats of all groups. The myocardial infarct size induced by I/R protocol was significantly higher in hearts of BET-exposed male rats compared to VEH-exposed male rats (VEH: 29.17 ± 9.88% of area at

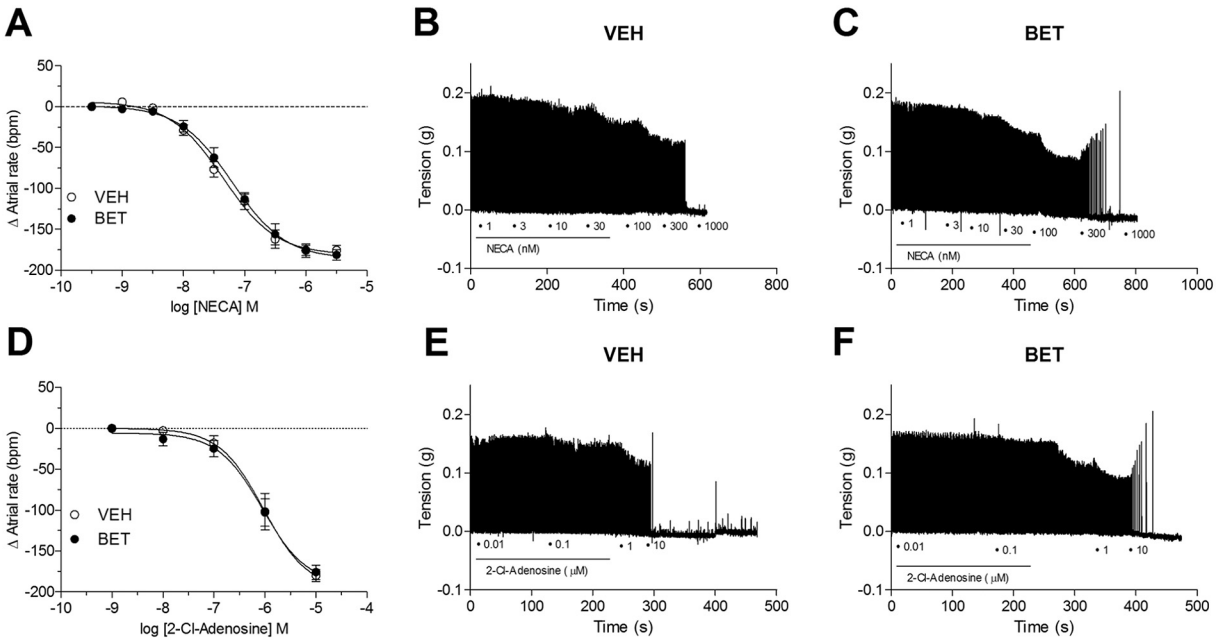

Fig. 2. *In vitro* RA chronotropic responses to non-metabolisable adenosine receptor agonists NECA and 2-Cl-adenosine. Representative trace-recordings of the negative chronotropic effect of NECA (A and B) and 2-Cl-adenosine (D and E) in RA from VEH and BET-exposed male rats (tension and time scales arbitrary set to zero). In C and F the mean concentration-response curves of NECA and 2-Cl-adenosine are presented, respectively. Data in C and E represent mean ± S.E.M. from 4 VEH- and 6 BET-exposed rats from different litters.

risk, $n = 5$ *vs* BET: 62.90 ± 3.17% of area at risk, $n = 7$; $p \leq 0.05$, Student's *t*-test; Fig. 4A).

No difference in the myocardial infarct size was observed in hearts from BET- and VEH-exposed female rats (VEH: 16.16 ± 4.6% of area at risk, $n = 6$vs BET: 17.99 ± 5.42% of area at risk, $n = 6$; $p \geq 0.05$, Student's *t*-test; Fig. 4C). Representative images of TTC-stained heart slices from male and female rats from the different groups are shown in Fig. 4B and D, respectively.

Hearts from VEH- and BET-exposed rats freshly harvested and not subjected to the Langendorff protocol presented no infarcted areas ($n = 4$ each group; data not shown).

4. Discussion

Excessive prenatal glucocorticoid exposure has been shown to impair fetal development (Reynolds, 2013). Accordingly, the offspring from BET-exposed pregnant rats had a 15% growth reduction at birth, a marker of adverse intrauterine environment ("programming"). This result shows that our protocol was able to induce fetal programming and is in line with the reported growth retardation effects of prenatal glucocorticoid exposure described in preclinical models (O'Regan et al., 2008).

BET-programmed male rats presented significant cardiac dysfunction encompassing impaired functioning of SAN and increased susceptibility to myocardial ischemic damage. Importantly, all these changes were observed before the onset of hypertension, suggesting them as contributors to the genesis of diseases rather than consequences of established dysfunctional states commonly seen at adulthood. The SAN from BET-programmed male rats fire at a high frequency and it's *in vitro* response to chronotropic agents adenosine and noradrenaline was shown to be impaired. Unfortunately, no attempt was made to evaluate whether the impaired chronotropic responses to adenosine and noradrenaline are present *in vivo* and we recognize this is a limitation of our study.

Activation of β-adrenoceptors on SAN pacemaker cells and the associated increase the HR is a mechanism of sympathetic nervous system regulation of hemodynamic homeostasis, thus, the 2.5-fold lower sensitivity of SAN to noradrenaline is an important observation. This result seems to contrast the reported increase in the β-adrenoceptor expression and signaling in the fetal hearts following prenatal glucocorticoid exposure in late gestation, however, the long-term maintenance of the modifications described by Slotkin et al. (1994) was not reported. Glucocorticoid-induced programming is also accompanied by a decreased sensitivity to α_1-adrenoceptor-mediated noradrenaline-induced contraction of mesenteric microvessels in lambs (Segar et al., 2006). The combined reduction of adrenergic control of HR and of peripheral vasoconstriction could be responsible for the increased sympathetic tonus uncovered previously in dexamethasone-programmed lambs (Segar et al., 2006).

The reduced negative chronotropic effect of adenosine in RA from BET-exposed male rats (≈ 12-fold reduction) is striking. Extracellular adenosine reduces HR by decrease in the frequency of spontaneous firing of SAN pacemaker cells through adenosine A1 receptor (A1R) activation (Tawfik-Schlieper et al., 1989) and any reduction of A1R

Table 3
Basal functional parameters of Langendorff-perfused hearts from prenatally VEH- and BET-exposed rats.

Male			Female		
	VEH ($n = 5$)	BET ($n = 7$)		VEH ($n = 6$)	BET ($n = 6$)
HR (bpm)	270.0 ± 33.7	282.0 ± 12.0	HR (bpm)	291.0 ± 31.5	291.00 ± 21.9
LVDP (mmHg)	34.1 ± 7.9	33.4 ± 5.5	LVDP (mmHg)	39.6 ± 10.5	42.23 ± 9.2
LVEDP (mmHg)	13.4 ± 5.2	10.5 ± 5.9	LVEDP (mmHg)	10.2 ± 7.0	11.21 ± 8.9
+ dp/dt (mmHg/s)	361.7 ± 57.1	526.1 ± 81.4	+ dp/dt (mmHg/s)	556.2 ± 178.6	598.3 ± 98.6
− dp/dt (mmHg/s)	− 235.0 ± 32.6	− 352.0 ± 61.7	− dp/dt (mmHg/s)	− 416.8 ± 131.8	− 409.1 ± 40.6

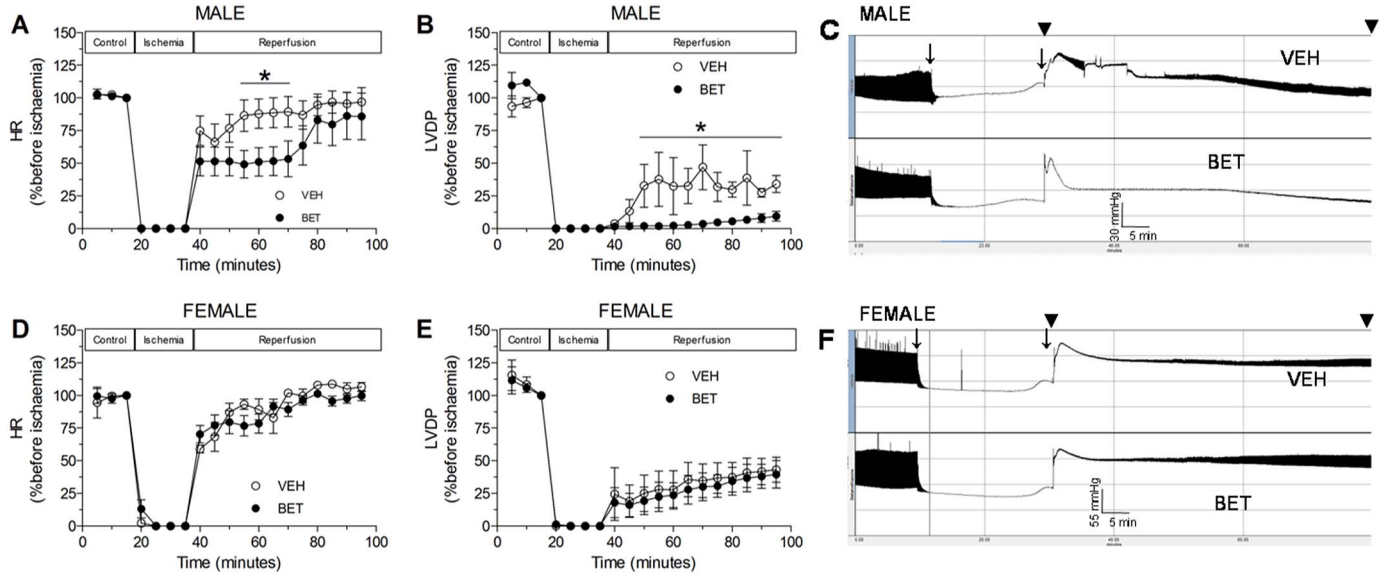

Fig. 3. Male-specific impairment of *in vitro* heart functional recovery of hearts from antenataly BET-exposed rats. A and B) HR and LVDP recovery of hearts from male (A and B) or female (D and E) rats during the I/R protocol. Original representative trace-recordings of left ventricular pressure measured by intraventricular saline-filled latex balloon in hearts from VEH- and BET-exposed male (C) and female (F) rats are shown. The arrows in (C) and (F) indicate the 20 min ischemia and the arrowheads the 1 h reperfusion period. Data represent mean ± S.E.M. from 5 to 7 rats from different litters. *$p \leq 0.05$ *vs* BET-exposed rats (Student's *t*-test).

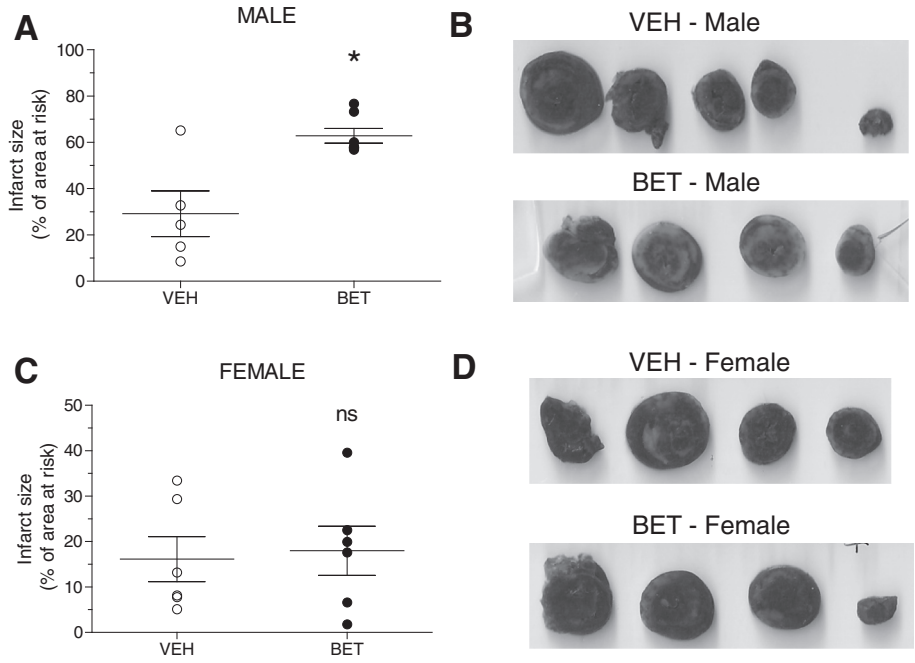

Fig. 4. Antenatal BET exposure engenders male-specific increased myocardial ischemia susceptibility in an *in vitro* infarct model. Myocardial infarct size from male (A) and female (C) VEH- and BET-exposed rats subjected to *in vitro* I/R protocol. The myocardial infarct area from different rats are shown as individual symbols and large traces and whiskers represent mean and the S.E.M., respectively. Representative images from TTC-stained heart slices from VEH- and BET-exposed male (B) and female (D) rats. Data from 5 to 7 rats from different litters. *$p \leq 0.05$ and ns: non-significant, $p > 0.05$ *vs* VEH-exposed rats (Student's *t*-test).

expression by SAN would impact the adenosine-induced chronotropic effect. Nevertheless, we think our results are not explained by a decreased A1R expression in SAN cells once in this scenario the chronotropic effects of NECA and 2-Cl-adenosine should be decreased as well.

The negative A1R effects on the spontaneous SAN firing occurs by activation of inwardly rectifying potassium channels (Kir3.4) *via* βγ G protein subunits (Belardinelli et al., 1995), therefore, changes in activity/expression of these signaling proteins would reduce the negative chronotropic effect of adenosine. Nevertheless, our data seems to exclude impaired intracellular signaling as a determinant of decreased adenosine effect as the negative chronotropism to acetylcholine, that utilizes the same signal transduction pathway employed by adenosine, was not modified at all (Belardinelli et al., 1995; Wang et al., 2013). As the chronotropic effects of NECA and 2-Cl-adenosine, two adenosine receptor agonists with reduced susceptibility to adenosine metabolism enzymes (Brown et al., 1982; Meester et al., 1998; Kenakin, 1982), were not changed it is suggested that an increased adenosine metabolism contributes, at least in part, to the blunted adenosine effects in the heart of BET-exposed male rats.

It's known that extracellular adenosine concentrations are tightly regulated by a complex array of enzymes such as equilibrative (ENT1-4) and concentrative nucleoside transporters (CNT1-3), adenosine kinase and adenosine deaminase (Fredholm et al., 2011). In the heart, adenosine metabolism by ENT1-2 (Rose et al., 2010), adenosine deaminase (Choong and Humphrey, 1987; Willems et al., 2006) and adenosine kinase (Fassett et al., 2011; Kroll et al., 1993) have been shown to control the extracellular adenosine levels and the anti-ischemic effect afforded by adenosine. In this scenario, it's of note that heart-specific

glucocorticoid receptor knockout mice have altered cardiac expression of adenosine kinase and adenosine deaminase (Oakley et al., 2013) suggesting their expression are under control of glucocorticoid action. At this time, however, we are not able to ascertain the apparent increase in the adenosine metabolism to any specific adenosine metabolism protein.

The putative increased cardiac adenosine metabolism could explain most of the differences in myocardial phenotype from BET-exposed rats observed in this study. As reported before the *in vivo* and *in vitro* HR is increased in A1R knockout mice suggesting spontaneous SAN rate is under adenosine control *via* A1R activation (Kirchhof et al., 2003; Neumann et al., 2003; Yang et al., 2009).

The basal firing rate of SAN is driven by the spontaneous plasma membrane depolarization through HCN4 sodium channels that carry the funny currents (Barbuti and DiFrancesco, 2008). The interstitial adenosine levels in rat perfused working hearts was estimated to be in the 0.3–0.5 μM (Fenton et al., 1990; Harrison et al., 1998), a concentration range showed to inhibit the funny current and decrease by 19% the firing rate from dog SAN pacemaker cells *via* A1R activation (Zaza et al., 1996). It's expected, therefore, that reduced extracellular adenosine would increase the SAN firing frequency.

Interestingly, albeit *in vitro* spontaneous SAN rate was increased by 12% neither the Langendorff HR nor the *in vivo* HR were changed in male rats exposed *in utero* to BET suggesting that compensatory mechanisms may be acting to avoid excessive HR in the whole heart scenario or alternatively, the existence of enough physiological dynamic reserve in the absence of an imposed stressor. In accordance, the basal HR from glucocorticoid-programmed rats was shown to be similar to control non-programmed rats in most of studies and an exacerbated HR response from rats exposed *in utero* to dexamethasone was uncovered only when exposed to mild stress (O'Regan et al., 2008). Whether the reduced SAN sensitivity to NA emerges as a possible contributor to this putative compensatory mechanism is not known. Importantly, a potential increased HR of BET-exposed male rats may be masked by our hemodynamic recording protocol done in the light phase of photoperiod as it was shown the effect of A1R on rodent HR is higher at night (Yang et al., 2009; Yang et al., 2007).

Albeit glucocorticoid administration to adult rats has been shown to protect isolated hearts from ischemia-induced injury (Xu et al., 2011; Xue et al., 2014) increased myocardial lesion was observed in animal models of fetal programming in which glucocorticoids were shown to play a role (Elmes et al., 2007; Lawrence et al., 2008). In fact, *in utero* BET exposure doubled the myocardial ischemic area, an effect associated with poor functional LV recovery. In addition to chronotropic actions in the heart myocardial adenosine receptors activation is an important protective mechanism against injury induced by ischemia (Liang and Jacobson, 1999). Extracellular adenosine levels in the heart increase up to 2000% during severe hypoxia (Edlund et al., 1983) and adenosine receptors activation limits the size of ischemic injury in the heart (Liang and Jacobson, 1999). These evidences concerning the role of adenosine in cardioprotection favor the hypothesis that the impaired adenosine signaling characterized in this study could contribute to the increased ischemia susceptibility of BET-exposed myocardium.

Impaired cardiac adenosine signaling resulting from increased adenosine metabolism is not unprecedented. Diabetic rats show increased susceptibility to cardiac ischemia parallel to impaired adenosine ischemic preconditioning, a dysfunction reversed by ENT1/4 blocker dipyridamole (Sharma et al., 2013). Overall, our results suggest the changes in SAN responses to adenosine and the higher susceptibility to myocardial ischemia of BET-programmed rats to be dependent on decreased adenosine signaling in the heart.

4.1. Gender-specific effects of in utero BET administration

Gender-specific effects of fetal programming on behavioral (Mueller and Bale, 2007, 2008) and cardiovascular parameters were described previously and shown to be dependent on fetal programming protocol employed (O'Regan et al., 2004).

We observed a clear male-over-female effect of glucocorticoid exposure on cardiac parameters employing the BET as programming stimulus as all the dysfunctions of antenatal BET exposure on male cardiac function were not shared by female rats. It's known that male, but not female rat fetuses, face an increase in blood testosterone (testosterone surge) between gestational days 17–19 which is important to acquisition of male reproductive behavior (Ward and Weisz, 1980; Weisz and Ward, 1980). Interestingly, androgens were shown to induce mouse embryonic stem cell differentiation to cardiomyocytes and to contribute to the maintenance of sexual dimorphism of myocardial structure and metabolism suggesting a role in the cardiac development as well (Goldman-Johnson et al., 2008; Ikeda et al., 2005; Koenig et al., 1982). As intrauterine dexamethasone exposure blunt the testosterone surge (Lalau et al., 1990) and interferes with the cardiac protein expression (Langdown et al., 2001; 2003), we suggest reduced intrauterine testosterone exposure as a putative mechanism contributing to the gender-specific cardiac dysfunctional state observed in BET-exposed male progeny. This hypothesis is in line with a previous study that found evidence of reduced testosterone surge in male progeny prenatally exposed to BET using the same treatment protocol employed by us (Piffer et al., 2009b).

The male selectivity of cardiac effects of BET-programming observed in our studies could be explained at some degree by a differential gender importance of adenosine control of cardiac physiology. In fact, although adenosine arrested the RA of both sexes *in vitro* the potency of adenosine effects was 2.5-fold higher in male atrium. Additionally, under basal conditions adenosine seems to play a more influential role in male but not female HR control in mice (Yang et al., 2007; 2009). A gender-dependence of adenosine effect in the heart cannot explain the differential ischemia susceptibility of BET-exposed rats as the adenosine-mediated cardioprotection was shown to be important in male and female hearts (Lankford et al., 2006).

In spite of we have no an explanation to the observed gender-specific effects of prenatal BET exposure it's noteworthy that 30-day old female BET-exposed rats recovered the body weight to normal levels as compared to age-matched VEH-exposed female rats. Whether the catch-up growth of female rats during the first post-natal month prevent the cardiac effects of BET exposure merits further investigation. It's noteworthy, however, that post-natal growth seems to reduce the rate of death from ischemic heart disease in adult men of low birth weight (Barker et al., 1989).

Altogether, the coincident reduced adenosine effect in the atrium and increased ischemia susceptibility in male *versus* female heart it's tempting to suggest the decreased adenosine signaling in the heart as a contributor factor of gender-specific effect of BET.

As increased myocardial susceptibility to ischemia is a hallmark of other models of intrauterine programming (Lawrence et al., 2008; Bae and Zhang, 2005; Li et al., 2003; Xue and Zhang, 2009) the investigation of adenosinergic system functioning in these models would allow the addressing of whether an impaired adenosine signaling is specific to prenatal glucocorticoid-exposure or is a part of a set of changes accompanying the fetal programming process.

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Conflict of interest

The authors declare no conflicts of interest.

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