

The Journal of Physiology

<http://jp.msubmit.net>

JP-RP-2016-273393R1

Title: SILDENAFIL THERAPY FOR FETAL CARDIOVASCULAR DYSFUNCTION DURING HYPOXIC DEVELOPMENT: STUDIES IN THE CHICK EMBRYO

Authors: Nozomi Itani
Katie Skeffington
Christian Beck
Dino Giussani

Author Conflict: No competing interests declared

Author Contribution: Nozomi Itani: Conception and design; Collection and assembly of data; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required) Katie Skeffington: Conception and design; Final approval of manuscript (required) Christian Beck: Conception and design; Final approval of manuscript (required) Dino Giussani: Conception and design; Financial Support; Data analysis and interpretation; Manuscript Writing; Final approval of manuscript (required)

Running Title: Sildenafil and fetal vascular function

Dual Publication: No

Funding: British Heart Foundation (BHF): Dino Giussani, PG/10/99/28656 and FS/12/74/29778

**SILDENAFIL THERAPY FOR FETAL CARDIOVASCULAR DYSFUNCTION
DURING HYPOXIC DEVELOPMENT: STUDIES IN THE CHICK EMBRYO**

Nozomi Itani¹, PhD; Katie L. Skeffington¹, MA; Christian Beck¹, BSc;

Dino A. Giussani¹, PhD

*¹Department of Physiology, Development & Neuroscience, University of Cambridge,
Downing Street, Cambridge, CB2 3EG, UK*

Running title: Sildenafil and fetal vascular function

Journal: *Journal of Physiology*

Key words: Fetus, Sildenafil, Endothelial function

Correspondence: Prof. Dino A Giussani
Department of Physiology, Development & Neuroscience
University of Cambridge, CB2 3EG
Tel: +44 1223 333894
Fax: +44 1223 333840
E-mail: dag26@cam.ac.uk

Table of contents category: Cardiovascular

KEY POINTS SUMMARY

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22

- Common complications of pregnancy, such as chronic fetal hypoxia, trigger a fetal origin of cardiovascular dysfunction and programme cardiovascular disease in later life;
- Sildenafil treatment protects placental perfusion and fetal growth. However, whether the effects of sildenafil transcend effects on the placenta to affect the fetus is unknown;
- Using the chick embryo model, here we show that sildenafil treatment directly protects the fetal cardiovascular system in hypoxic development, and that the mechanisms of sildenafil protection includes reduced oxidative stress and increased nitric oxide bioavailability;
- Sildenafil does not protect against fetal growth restriction in the chick embryo, supporting the idea that the protective effect of sildenafil on fetal growth reported in mammalian studies, including humans, is secondary to improved placental perfusion.
- Therefore, sildenafil may be a good candidate for human translational antioxidant therapy to protect the chronically hypoxic fetus in adverse pregnancy.

23
24

ABSTRACT

25 There is a need for developing clinically translatable therapy for preventing fetal origins
26 of cardiovascular disease in pregnancy complicated by chronic fetal hypoxia. Evidence
27 shows that sildenafil protects placental perfusion and fetal growth. However, whether
28 beneficial effects of sildenafil transcend onto the fetal heart and circulation in
29 complicated development is unknown. We isolated the direct effects of sildenafil on the
30 fetus using the chick embryo and hypothesised that sildenafil also protects fetal
31 cardiovascular function in hypoxic development. Chick embryos (n=11 per group) were
32 incubated in normoxia or hypoxia (14% O₂) from day 1 and treated with sildenafil
33 (4mg/kg/day) from day 13 of the 21-day incubation. Hypoxic incubation increased
34 oxidative stress (4-hydroxynoneal, 141.1 ± 17.6% of normoxic control), reduced
35 superoxide dismutase (60.7 ± 6.3%), increased phosphodiesterase type 5 expression
36 (167 ± 13.7%) and decreased nitric oxide bioavailability (54.7 ± 6.1%) in the fetal heart,
37 and promoted peripheral endothelial dysfunction (70.9 ± 5.6 AUC of normoxic control;
38 all *P* < 0.05). Sildenafil treatment after onset of chronic hypoxia prevented the increase
39 in phosphodiesterase expression (72.5 ± 22.4), protected against oxidative stress (94.7 ±
40 6.2) and normalised nitric oxide bioavailability (115.6 ± 22.3) in the fetal heart, and
41 restored endothelial function in the peripheral circulation (89.8 ± 2.9). Sildenafil
42 protects the fetal heart and circulation directly in hypoxic development via mechanisms
43 including decreased oxidative stress and enhanced nitric oxide bioavailability. Sildenafil
44 may be a good translational candidate for human antioxidant therapy to prevent fetal
45 origins of cardiovascular dysfunction in adverse pregnancy.

46

47

Abbreviations

48 3-NT, 3-nitrotyrosine; 4-HNE, 4-hydroxynoneal; ACh, acetylcholine; COX,
49 cyclooxygenase; GMP, guanosine monophosphate; GPx, glutathione peroxidase; H,
50 hypoxic; HS, hypoxic sildenafil; IUGR, intrauterine growth restriction; N, normoxic;
51 NO, nitric oxide; NOx, nitric oxide species; NS, normoxic sildenafil; PDE5,
52 phosphodiesterase type 5; PE, phenylephrine; SNP, sodium nitroprusside; SOD,
53 superoxide dismutase; ROS, reactive oxygen species.

54

INTRODUCTION

55

56 It is widely accepted from data derived from humans and animal models that adverse
57 conditions during pregnancy can trigger fetal growth restriction and an early origin of
58 cardiovascular disease (Barker *et al.*, 1993; Gluckman *et al.*, 2008; Giussani & Davidge,
59 2013). Chronic fetal hypoxia is common during adverse pregnancy (Giussani, 2016) and
60 independent studies have shown that chronic fetal hypoxia can trigger cardiovascular
61 dysfunction in the offspring secondary to oxidative stress (Giussani *et al.*, 2012;
62 Patterson *et al.*, 2012; Thompson & Al-Hasan, 2012; Giussani & Davidge, 2013). For
63 instance, hypoxic pregnancy in rats increased levels of oxidative stress in the fetal heart
64 and vasculature, setting cardiac sympathetic dominance and endothelial dysfunction in
65 the adult offspring; maternal treatment with the antioxidant vitamin C was protective
66 (Giussani *et al.*, 2012; Kane *et al.*, 2013). Although such studies provided proof-of-
67 principle to support the idea that maternal antioxidants protect against fetal origins of
68 cardiovascular dysfunction in the chronically hypoxic fetus, only high doses of vitamin
69 C incompatible with human treatment were effective. Further, in these studies maternal
70 antioxidant therapy was administered from the onset of chronic fetal hypoxia, limiting
71 their human translational capacity (Giussani *et al.*, 2012; Kane *et al.*, 2013). Clinically,
72 diagnosis prior to treatment is necessary, therefore maternal antioxidant treatment
73 following established chronic fetal hypoxia would provide a better translational study
74 design.

75

76 One possible alternative candidate therapy is sildenafil, the selective inhibitor of cyclic
77 guanosine monophosphate (GMP)-specific phosphodiesterase type 5 (PDE5). Sildenafil
78 has direct antioxidant properties (Koupparis *et al.*, 2005) and by preventing the
79 hydrolysis of cyclic GMP by PDE5, it additionally increases the bioavailability of cyclic
80 GMP, a downstream secondary messenger of the potent vasodilator nitric oxide (NO,
81 Francis & Corbin, 2003). Sildenafil treatment in human, ovine and murine pregnancy
82 complicated by fetal growth restriction improved placental perfusion, increasing
83 umbilical blood flow and protecting fetal growth (Satterfield *et al.*, 2010; von
84 Dadelszen *et al.*, 2011; Stanley *et al.*, 2012; Dilworth *et al.*, 2013). Therefore, a large

85 multicentre international scheme was recently launched to determine the efficacy of
86 sildenafil as candidate human clinical intervention for fetal growth restriction
87 (Ganzevoort *et al.*, 2014). However, whether the positive effects of sildenafil transcend
88 those on placental perfusion and fetal growth onto beneficial effects on the fetal
89 cardiovascular system is completely unknown. Equally important, whether sildenafil
90 has any potential adverse effects on fetal cardiovascular function in addition to effects
91 on the maternal and/or placental physiology in healthy or complicated development is
92 unclear.

93

94 Therefore, this study isolated the effects of sildenafil on the fetal cardiovascular system
95 using the chick embryo, the only established animal model in which the direct effects on
96 the fetal heart and circulation of potential therapy can be investigated, independent of
97 effects on the mother and/or the placenta. The study tested the hypothesis that sildenafil
98 treatment has direct beneficial effects on the fetal cardiovascular system in development
99 complicated by chronic fetal hypoxia. In addition, we proposed that mechanisms of
100 protection include reduced oxidative stress with enhanced NO bioavailability. The
101 hypothesis was tested by investigating the effects of normoxic or hypoxic incubation of
102 chick embryos with or without sildenafil treatment on fetal growth, fetal peripheral
103 vascular reactivity and on molecular indices of oxidative stress, antioxidant capacity and
104 NO bioavailability. Treatment of chick embryos with sildenafil started at day 13 of
105 incubation, equivalent to ca. 25 weeks of gestation in human pregnancy, a gestational
106 age at which human fetal growth restriction can be reliably diagnosed.

107

METHODS

108 **Ethical Approval**

109 All procedures were performed under the UK Animals (Scientific Procedures) Act 1986
110 and were approved by the Ethical Review Committee of the University of Cambridge,
111 as described in the Editorial by Grundy (2015).

112

113 **Animals**

114 Fertilised Bovans Brown eggs (Medeggs, Norfolk, UK) were weighed and incubated
115 under normoxic (21% O₂) or hypoxic (14±0.5% O₂) conditions (37.9°C, 45% humidity,
116 12:12h light:dark cycle, automatic rotation every hour, Mod-75A equipped with
117 electronic servo-controlled humidity system HS-Auto-3.5L, Marsalles, Barcelona,
118 Spain) from day 1. The levels of oxygen, humidity and temperature inside the
119 incubators were continuously monitored (DD103 DrDAQ Oxygen Sensor, Pico
120 Technology, St. Neots, UK).

121

122 **Dose of sildenafil**

123 In clinical studies in which sildenafil has been administered to pregnant women, the
124 dose varies between 0.86-3.43 mg/kg/d (Samangaya *et al.*, 2009; von Dadelszen *et al.*,
125 2011) assuming a 60 kg body weight at pre-conception and a weight gain of 10 kg by 25
126 weeks of gestation (Bhattacharya *et al.*, 2007; Fraser *et al.*, 2010). The dose of chronic
127 sildenafil treatment used in animal studies varies between 0.5-90 mg/kg/d (Refuerzo *et*
128 *al.*, 2006; Sanchez-Aparicio *et al.*, 2008). Notably, the metabolism of sildenafil also
129 differs between species (Walker *et al.*, 1999) and no pharmacokinetic study of sildenafil
130 in the chicken has been previously reported. Collectively, from previous human and
131 animal data available, a 4 mg/kg/d dose regimen was chosen for the present study as a
132 dose that is human clinically as well as scientifically relevant. Therefore, chick embryos
133 were treated with sildenafil (4mg/kg/d, Sildenafil citrate salt, Sigma-Aldrich, UK) or
134 vehicle (100µl water) from day 13 to day 18 of incubation. Sildenafil was injected daily
135 into the air cell onto the chorioallantoic membrane via a 1 mm hole in the eggshell of
136 normoxic or hypoxic eggs. N = 11 eggs were used per group (normoxic control,

137 hypoxic control, hypoxic sildenafil, normoxic sildenafil). The hole was covered with
138 tape at all other times. All treatment procedures were performed under sterile
139 conditions.

140

141 **Haematocrit and growth analysis**

142 On day 19 of the 21 day incubation period, embryos underwent euthanasia by
143 decapitation and immediately *post mortem* the weight of the embryo, yolk, extra-
144 embryonic membranes, chorioallantoic fluid and the shell was recorded and expressed
145 as percentages of the egg weight on day 19 to determine how much resource was turned
146 into fetal body mass within each egg. Blood was collected in micro-haematocrit tubes
147 (Vitrex, Modulohm, Denmark) directly from the heart and the haematocrit was
148 determined in duplicate. Body length was measured by placing the ends of a digital
149 calliper on top of the head and the base of the tail. The heart, brain, liver, lungs and the
150 kidneys were dissected and weighed. A section of the third order femoral artery was
151 dissected for vascular reactivity analysis. The heart was snap frozen in liquid nitrogen
152 and stored at -80°C until molecular analysis.

153

154 **Molecular studies in the chick embryo heart**

155 Alterations in the pro-oxidant indices 3-nitrotyrosine (3-NT) and 4-hydroxynoneal (4-
156 HNE), in the antioxidant enzymes superoxide dismutase (SOD), catalase and
157 glutathione peroxidase (GPx), and in the level of NO species (NOx) were determined in
158 the chick embryo heart at day 19 of incubation. In addition, changes in the cardiac
159 expression of PDE5 were determined to validate the effect of sildenafil treatment.

160

161 The expression of 3-NT, 4-HNE and SOD, the activity of catalase and the levels of NOx
162 were determined by commercial assay kits according to the manufacturers' instructions
163 (3-NT: ab116691, Abcam, Cambridge, UK., 4-HNE: E12H0203, AMS biotechnology,
164 Abington, UK., SOD: Sigma-Aldrich, UK., Catalase: 707002 and NOx: 78001, Cayman
165 Chemical Company, MI, USA).

166

167 The cardiac expression of GPx and PDE5 was determined by Western Blot. Frozen
168 chick hearts (25mg) were powdered on dry ice and homogenized in 250µl of ice-cold
169 lysis buffer (HEPES:50mM, NaCl:150mM, Triton-X100:1%, Na₃VO₄:1mM,
170 NaF:30mM, Na₄P₂O₇:10mM, EDTA:10mM, Protease inhibitor cocktail III [Calbiochem,
171 Nottingham, UK]) in a microtube containing 1.4 mm ceramic beads (Lysing Matrix D,
172 MP biomedical). The protein concentration of lysates was determined using the
173 copper-Bicinchoninic assay (Smith *et al.*, 1985). Protein samples were diluted with 5x
174 Laemmli's buffer (sodium dodecyl sulphate (SDS):2%, Tris-HCl (pH 6.8):62.5mM,
175 glycerol:10%, dithiothreitol:100mM, bromophenol blue) then standardized to a protein
176 concentration of 1 mg/ml by further addition of 1 x Laemmli's buffer. Total protein (10
177 µg) from each sample (n = 6 per treatment group) was separated on SDS-PAGE gel
178 electrophoresis along with a pre-stained molecular weight marker (PageRuler Plus
179 Prestained Protein Ladder 10-250kDa, Thermo Scientific, Waltham, USA), then
180 transferred immediately to a Polyvinylidene difluoride Immobilon-P membrane (PVDF,
181 Millipore, Billerica, MA, USA). The membrane was incubated in blocking buffer before
182 incubation in primary antibodies (GPx, ab22604; PDE5, ab64179; Abcam) overnight at
183 4°C. Following primary antibody incubation the membranes were incubated with the
184 secondary antibody (Peroxidase-AffiniPure Donkey Anti-Rabbit IgG (H+L), 1:10000 in
185 PBS with 1% marvel and 0.1% Tween 20, Jackson ImmunoResearch laboratories, PA,
186 USA) and immunoreactivity was measured using West Pico chemiluminescent substrate
187 (Thermo Scientific). The intensity of the bands were analysed with the AlphaEase
188 imaging software (Alpha Innotech, San Leandro, CA, USA). Following the protein
189 detection, the membrane was stained with 0.1% Coomassie R-250 and the intensity of
190 the Coomassie staining for each lane was analysed (Welinder & Ekblad, 2011). The
191 expression level of the target protein in each sample was normalised to the Coomassie
192 staining of the same sample for a loading control.

193

194 **Functional peripheral vascular reactivity using *in vitro* wire myography**

195 Constrictor and dilator function of the peripheral resistance vasculature was assessed
196 using a microvascular myograph (Wire Myograph System 610M; DMT, Aarhus,

197 Denmark) as previously described (Itani *et al.*, 2016). Briefly, a third order femoral
198 artery was dissected at day 19 of incubation and mounted in a chamber containing
199 Krebs's buffer. Vascular constrictor capacity was assessed with increasing doses of K⁺
200 solutions (16.74 – 250 mM) and of phenylephrine (PE, 10⁻⁸ – 10⁻⁴ M). The response to
201 K⁺ was normalised to the diameter of the vessel (mN/mm/μm/1000). The response to
202 phenylephrine was normalised to the constrictor response to 125mM K⁺ achieved by the
203 same vessel (% K⁺125). Vasodilator responses to cumulative doses of sodium
204 nitroprusside (SNP, 10⁻¹⁰ – 10⁻⁴ M) and of acetylcholine (ACh, 10⁻⁹ – 10⁻⁵ M) were
205 assessed after pre-constricting the vessel with a sub-optimal dose of potassium. The
206 partial contributions of endogenous NO-dependent and NO-independent mechanisms to
207 the vasorelaxation were determined by repeating the ACh dose response curve after
208 incubating the vessel with L-NAME (10⁻⁵ M, 10min) and calculating the area under the
209 curves (Herrera *et al.*, 2010; Itani *et al.*, 2016). The sensitivity (pD2) to ACh was
210 defined as $-\log_{10}(\text{EC}_{50})$. LabChart was used for data acquisition and analysis of the *in*
211 *vitro* wire myography data (LabChart 6.0, Powerlab 8/30; AD Instruments, Chalgrove,
212 UK).

213

214 **Statistical analysis**

215 All data are expressed as mean ± S.E.M. Data were checked for Gaussian distribution
216 using the D'Agostino-Pearson normality test. Statistical comparisons were made using
217 Two-way ANOVA, with the Bonferroni *post hoc* test where a significant interaction
218 was detected. For all comparisons, statistical significance was accepted when P<0.05.
219 (Graphpad prism version 5.00, *Graphpad Software, Inc.* San Diego, USA).

220

RESULTS

221 **Haematocrit and fetal growth**

222 Incubation under hypoxic conditions from day 1 significantly increased haematocrit in
223 the chick embryo by day 19 (Figure 1A). Exposure to hypoxia throughout development
224 reduced the body weight (Figure 1B) which persisted when the body weight was
225 normalised to the egg weight at the start of incubation (N: 41.3 ± 1.0 , H: $29.0 \pm 1.3^*$, HS:
226 $32.0 \pm 2.2^*$, NS: 43.9 ± 1.3 g/g, $P < 0.05$; *effect of hypoxia). Hypoxia affected the body
227 weight more severely than the body length (N: 68.1 ± 1.7 , H: $63.2 \pm 1.0^*$, HS: 65.9 ± 0.9 ,
228 NS: 70.8 ± 0.6 mm, $P < 0.05$; *versus N) of the embryo. Consequently, hypoxic embryos
229 had a lower BMI (N: 5.3 ± 0.2 , H: $4.6 \pm 0.1^*$, HS: $4.5 \pm 0.2^*$, NS: 5.1 ± 0.1 kg/m², $P < 0.05$;
230 *effect of hypoxia). The brain weight was reduced in the hypoxic embryo (N: 0.84 ± 0.01 ,
231 H: $0.73 \pm 0.02^*$, HS: $0.74 \pm 0.01^*$, NS: 0.84 ± 0.01 g, $P < 0.05$; *effect of hypoxia),
232 however this was not proportional to the reduction in their body size and thus, the
233 relative brain weight was increased (Figure 1C). In addition, calculation of resource
234 partitioning revealed less resource attributed to embryonic mass in hypoxic incubations
235 (Figure 1D). Collectively, the data show that those embryos exposed to chronic hypoxia
236 were thin for their length and had relative brain sparing. Sildenafil treatment from day
237 13 of incubation had no effect on changes in fetal growth or brain sparing during either
238 hypoxic or normoxic incubation (Figure 1).

239

240 Exposure to chronic hypoxia from day 1 significantly reduced the weight of the heart,
241 lungs, liver and kidneys by day 19 in the chick embryo, and this was proportional to the
242 reduction in body size (Table 1). Sildenafil treatment in normoxic and hypoxic embryos
243 did not affect the absolute or relative weight of the lungs, liver or kidneys. However,
244 both absolute and relative heart weights were significantly reduced in normoxic
245 embryos treated with sildenafil, while the relative heart weight was also reduced in
246 hypoxic embryos treated with sildenafil (Table 1).

247

248 **Molecular studies in the chick embryo heart**

249 In the heart of chick embryos exposed to chronic hypoxia, the protein expression of
250 3-NT and 4-HNE was significantly elevated (Figure 2A and B). In addition, the
251 expression of SOD and the activity of catalase were both decreased in the hypoxic heart
252 (Figure 3A and B). Hypoxic incubation had no effect on the cardiac expression of GPx
253 (Figure 3C) but the total cardiac NOx concentration was reduced (Figure 3D). Sildenafil
254 treatment prevented the increase in 4-HNE but not 3-NT in the hypoxic embryo heart.
255 Cardiac NOx levels were restored by sildenafil treatment in the hypoxic embryo.
256 However the treatment significantly reduced the levels of cardiac NOx in normoxic
257 embryos. Sildenafil treatment had no significant effect on the expression of SOD or the
258 activity of catalase, however the protein expression of GPx in the heart of hypoxic
259 embryos was significantly elevated (Figure 2 and 3).

260

261 Compared to normoxic embryos, the protein expression of PDE5 in the heart was
262 significantly enhanced in hypoxic embryos treated with vehicle. Sildenafil treatment of
263 hypoxic embryos normalised the protein expression of cardiac PDE5 and sildenafil
264 treatment of normoxic embryos showed no effect on the protein expression of cardiac
265 PDE5 (Figure 4).

266

267 **Functional peripheral vascular reactivity using *in vitro* wire myography**

268 The femoral arterial segments displayed a dose-dependent relaxation in response to SNP
269 and ACh (Figures 5 and 6). The femoral arterial vascular response to SNP was not
270 significantly affected by hypoxic incubation or sildenafil treatment (Figure 6A). In
271 contrast, incubation under hypoxic conditions shifted the ACh relaxation curve to the
272 right, with vessels requiring higher concentrations of ACh to achieve similar relaxation
273 (Figure 5 and 6B). Consequently, compared to normoxic embryos, the sensitivity (pD₂)
274 of the vessels to ACh was significantly reduced in hypoxic embryos. In addition the
275 total relaxant capacity to ACh, measured as area under the curve (AUC), was
276 significantly reduced in the hypoxic embryo (Figure 6C). The ACh relaxant curve was
277 repeated in presence of a NO synthase blocker L-NAME to determine the partial

278 contributions of NO-dependent and NO-independent components of the vasodilation,
279 which revealed that the deficit in the total femoral vascular relaxation in response to
280 ACh in hypoxic embryos was primarily due to NO-independent mechanisms. Sildenafil
281 treatment in hypoxic embryos rescued the vasodilation both in terms of sensitivity and
282 total relaxation by significantly enhancing the NO-dependent component of the
283 vasodilation (Figure 6B and C). Sildenafil treatment in normoxic embryos did not affect
284 femoral vascular dilator function.

285

286 Relative to normoxic embryos, those incubated under hypoxic conditions displayed a
287 significantly enhanced maximal constrictor response to potassium (N: 2.4 ± 0.2 , H:
288 $4.4 \pm 0.9^*$, HS: $4.4 \pm 0.5^*$, NS: 2.5 ± 0.3 mN/mm/ μ m/1000) but constrictor responses to
289 phenylephrine were not affected (N: 116 ± 4 , H: 118 ± 4 , HS: 125 ± 10 , NS: $124 \pm 10\%$
290 K^+ 125 mM). Sildenafil treatment had no effect on femoral constrictor function.

DISCUSSION

291

292

293 The data in this study show that hypoxic incubation of the chick embryo led to an
294 increase in fetal haematocrit and promoted asymmetric fetal growth restriction by the
295 end of the incubation period. Hypoxic incubation increased levels of oxidative stress in
296 the fetal heart, reduced cardiac antioxidant defences, increased cardiac PDE5 expression,
297 decreased cardiac NO bioavailability and promoted endothelial dysfunction in
298 peripheral resistance vessels. Treatment of the chronically hypoxic chick embryo with
299 sildenafil long after the onset of chronic hypoxia prevented the increase in cardiac
300 PDE5 expression, protected against cardiac oxidative stress, normalised cardiac NO
301 bioavailability and restored peripheral endothelial function. Therefore, the data support
302 the hypothesis tested that sildenafil has direct beneficial effects on the fetal
303 cardiovascular system in development complicated by chronic fetal hypoxia.

304

305 In addition to its obvious advantages of higher throughput and lower cost over other
306 animal models, the chick embryo is the only established animal model that permits
307 isolation of the direct effects on the fetus of developmental hypoxia, oxidative stress
308 and/or treatment independent of additional effects of the experimental design on
309 maternal nutrition or changes in the placental and/or maternal physiology. The ontogeny
310 of cardiac development in the chicken is much more comparable to the human than the
311 rat or the mouse (Marcela *et al.*, 2012). Further, mechanisms underlying the control of
312 cardiovascular function in the chick embryo and the human fetus show many
313 similarities (Crossley & Altimiras, 2000; Mulder *et al.*, 2000; Ruijtenbeek *et al.*, 2002;
314 Giussani, 2016). Consequently, the chick embryo model has been used by independent
315 groups to isolate the effects on fetal growth and on fetal cardiovascular function of
316 development complicated by chronic fetal hypoxia (Ruijtenbeek *et al.*, 2000;
317 Dzialowski *et al.*, 2002; Rouwet *et al.*, 2002; Sharma *et al.*, 2006; Salinas *et al.*, 2010;
318 Giussani, 2016; Itani *et al.*, 2016).

319

320 Accumulating evidence shows that PDE5 is involved in many cardiac disease states
321 where perturbations in NO signalling is implicated (Kass *et al.*, 2007). The expression
322 of PDE5 is up-regulated in the left ventricle in patients with end-stage ischemic or
323 dilated cardiomyopathy (Pokreisz *et al.*, 2009) and in hypertrophied hearts (Nagendran
324 *et al.*, 2007). Importantly, the increase in myocardial PDE5 in the failing heart is
325 associated with increased levels of cardiac 3-NT and 4-HNE (Lu *et al.*, 2010). Chronic
326 hypoxia also stimulates a number of pro-oxidant pathways such as xanthine-oxidase
327 (Kane *et al.*, 2014), as well as consuming a number of antioxidant defences (Maiti *et al.*,
328 2006; Giussani & Davidge, 2013). In the present study, treatment with sildenafil
329 prevented the chronic hypoxia-induced increase in PDE5 and in 4-HNE, it increased
330 glutathione and restored NO bioavailability in the chick embryo heart. 4-HNE is a
331 major product of lipid peroxidation and it is formed via several ROS-dependent
332 pathways (Spickett, 2013). GPx is an established antioxidant enzyme (Masella *et al.*,
333 2005). Therefore, the restored levels of NOx and 4-HNE coupled with the enhanced
334 levels of GPx in the heart of sildenafil-treated hypoxic chick embryos support direct
335 antioxidant mechanisms of sildenafil on the fetal cardiovascular system.

336

337 Additional data presented in this study show that exposure to chronic hypoxia during
338 development leads to impaired dilation in the peripheral vasculature, predominantly
339 through NO-independent mechanisms. The binding of ACh to its trans-membrane
340 receptors on the endothelial cell increases the level of intracellular calcium (Ca^{2+}),
341 releasing arachidonic acid within the cell that is, in turn, converted into prostaglandins
342 by cyclooxygenases COX1 and COX2 (Bachschmid *et al.*, 2005). During chronic
343 hypoxia, arachidonic acid is preferentially converted into constrictor prostaglandins,
344 such as TXA_2 and $PGF_{2\alpha}$ via enhanced COX2 activity (Fike *et al.*, 2005; Wong *et al.*,
345 2009). It is well known that hypoxia at the tissue level leads to an increased generation
346 of ROS, particularly the superoxide anion ($\bullet O_2^-$) at the mitochondria (Chandel *et al.*,
347 1998; Becker *et al.*, 1999). $\bullet O_2^-$ readily combines with NO to limit its bioavailability
348 (Kissner *et al.*, 1997; Thakor *et al.*, 2010b). Therefore, chronic hypoxia shifts the
349 cardiovascular phenotype into one of oxidative stress as well as switching the
350 metabolism of arachidonic acid towards constrictor pathways (Fike *et al.*, 2005;

351 Delannoy *et al.*, 2010; Giussani, 2016). In the present study, treatment with sildenafil of
352 hypoxic chick embryos restored the endothelium-dependent relaxation of the femoral
353 artery by enhancing NO-dependent mechanisms. This agrees with the principal dilator
354 mechanism of action of sildenafil, enhancing downstream signalling of NO. The ratio of
355 $\bullet\text{O}_2^-:\text{NO}$ yields a vascular oxidant tone and we have shown that this is functional in fetal
356 life and that it can be manipulated in favour of dilation (Thakor *et al.*, 2010a; Thakor *et*
357 *al.*, 2010b; Giussani *et al.*, 2012; Kane *et al.*, 2014). Mechanisms in addition to
358 antioxidant properties underlying the beneficial effects of sildenafil in the chronically
359 hypoxic fetus therefore include inhibition of the degradation of cyclic GMP by PDE5,
360 enhancing the action of NO, thereby normalising the vascular oxidant tone and
361 endothelial function.

362

363 Other data in the present study show that the femoral maximal contractile response to
364 potassium was significantly enhanced in the chick embryo exposed to chronic hypoxia.
365 The hypoxia-induced increase in the femoral contractile capacity in the chronically
366 hypoxic chick embryo may be a direct effect of hypoxia and/or secondary to the known
367 effects of chronic hypoxia in promoting sympathetic hyperinnervation (Ruijtenbeek *et*
368 *al.*, 2000). The latter has also been associated with proliferation and differentiation of
369 vascular smooth muscle cells (le Noble *et al.*, 2000; Rouwet *et al.*, 2002). In the present
370 study, sildenafil treatment did not diminish the magnitude of the femoral constrictor
371 responses to either phenylephrine or to potassium, further supporting that the
372 mechanism of action mediating the improved peripheral vasodilation is via enhancing
373 NO-dependent actions rather than by depressing constrictor mechanisms.

374

375 In the present study, treatment with sildenafil of hypoxic chick embryos did not
376 improve the fetal growth restriction but brain sparing in growth-restricted fetuses was
377 preserved. Alterations in the ratio of $\bullet\text{O}_2^-:\text{NO}$ promoting a vascular oxidant tone may
378 have a significant effect on circulations which are particularly sensitive to NO, such as
379 the placental and umbilical vascular bed (Derks *et al.*, 2010; Thakor *et al.*, 2010a). In
380 support, a number of studies in humans and mammalian animal models have reported

381 possible protection by sildenafil against fetal growth restriction secondary to improved
382 placental perfusion in complicated pregnancy (Refuerzo *et al.*, 2006; Sanchez-Aparicio
383 *et al.*, 2008; Satterfield *et al.*, 2010; von Dadelszen *et al.*, 2011; Herraiz *et al.*, 2012;
384 Stanley *et al.*, 2012; Dilworth *et al.*, 2013). In the present study, sildenafil did not
385 prevent growth restriction in the chronically hypoxic chick embryo, supporting a
386 protective effect of sildenafil on fetal growth in mammalian species by improving
387 placental perfusion. Alternatively, it could be argued that sildenafil treatment in this
388 model of hypoxic development started too late following the onset of chronic fetal
389 hypoxia to prevent fetal growth restriction.

390

391 In conclusion, we have intertwined the use of the chick embryo with hypoxic incubation
392 to provide the first evidence that sildenafil has direct protective effects on the fetal heart
393 and vasculature, independent of the presence of a placenta. The mechanisms underlying
394 the protection conveyed by sildenafil on the fetal cardiovascular system include
395 inhibition of PDE5, increased antioxidant defences, diminished oxidative stress,
396 increased NO bioavailability and diminished NO-dependent endothelial dysfunction.
397 The protective effects of sildenafil on the cardiovascular system of the chronically
398 hypoxic fetus were evident even when sildenafil therapy was started long after the onset
399 of chronic hypoxia. The lack of a protective effect of sildenafil treatment on fetal
400 growth in the chick embryo during hypoxic development supports beneficial effects of
401 sildenafil on growth in IUGR fetuses of mammalian species, including humans, to be at
402 the level of the placenta. Future research will therefore need to consider direct and
403 indirect effects of sildenafil at the maternal, placental and fetal levels. However,
404 sildenafil offers to be a plausible candidate for human clinical translational therapy to
405 rescue adverse effects of pregnancy complicated by developmental hypoxia.

406

407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447

REFERENCES

- Bachschnid M, Schildknecht S & Ullrich V. (2005). Redox regulation of vascular prostanoid synthesis by the nitric oxide-superoxide system. *Biochem Biophys Res Commun* **338**, 536-542.
- Barker DJ, Osmond C, Simmonds SJ & Wield GA. (1993). The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. *BMJ* **306**, 422-426.
- Becker LB, vanden Hoek TL, Shao ZH, Li CQ & Schumacker PT. (1999). Generation of superoxide in cardiomyocytes during ischemia before reperfusion. *Am J Physiol* **277**, H2240-2246.
- Bhattacharya S, Campbell DM, Liston WA & Bhattacharya S. (2007). Effect of Body Mass Index on pregnancy outcomes in nulliparous women delivering singleton babies. *BMC Public Health* **7**, 168.
- Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC & Schumacker PT. (1998). Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc Natl Acad Sci U S A* **95**, 11715-11720.
- Crossley D & Altimiras J. (2000). Ontogeny of cholinergic and adrenergic cardiovascular regulation in the domestic chicken (*Gallus gallus*). *Am J Physiol Regul Integr Comp Physiol* **279**, R1091-1098.
- Delannoy E, Courtois A, Freund-Michel V, Leblais V, Marthan R & Muller B. (2010). Hypoxia-induced hyperreactivity of pulmonary arteries: role of cyclooxygenase-2, isoprostanes, and thromboxane receptors. *Cardiovasc Res* **85**, 582-592.
- Derks JB, Oudijk MA, Torrance HL, Rademaker CM, Benders MJ, Rosen KG, Cindrova-Davies T, Thakor AS, Visser GH, Burton GJ, van Bel F & Giussani DA. (2010). Allopurinol reduces oxidative stress in the ovine fetal cardiovascular system after repeated episodes of ischemia-reperfusion. *Pediatr Res* **68**, 374-380.
- Dilworth MR, Andersson I, Renshall LJ, Cowley E, Baker P, Greenwood S, Sibley CP & Wareing M. (2013). Sildenafil citrate increases fetal weight in a mouse model of fetal growth restriction with a normal vascular phenotype. *PLoS One* **8**, e77748.
- Dzialowski EM, von Plettenberg D, Elmonoufy NA & Burggren WW. (2002). Chronic hypoxia alters the physiological and morphological trajectories of developing chicken embryos. *Comp Biochem Physiol A Mol Integr Physiol* **131**, 713-724.

448 Fike CD, Kaplowitz MR, Zhang Y & Pfister SL. (2005). Cyclooxygenase-2 and an early stage of
449 chronic hypoxia-induced pulmonary hypertension in newborn pigs. *J Appl Physiol*
450 (1985) **98**, 1111-1118; discussion 1091.

451

452 Francis SH & Corbin JD. (2003). Molecular mechanisms and pharmacokinetics of
453 phosphodiesterase-5 antagonists. *Curr Urol Rep* **4**, 457-465.

454

455 Fraser A, Tilling K, Macdonald-Wallis C, Sattar N, Brion MJ, Benfield L, Ness A, Deanfield J,
456 Hingorani A, Nelson SM, Smith GD & Lawlor DA. (2010). Association of maternal weight
457 gain in pregnancy with offspring obesity and metabolic and vascular traits in
458 childhood. *Circulation* **121**, 2557-2564.

459

460 Ganzevoort W, Alfirevic Z, von Dadelszen P, Kenny L, Papageorgiou A, van Wassenaer-
461 Leemhuis A, Glud C, Mol BW & Baker PN. (2014). STRIDER: Sildenafil Therapy In
462 Dismal prognosis Early-onset intrauterine growth Restriction--a protocol for a
463 systematic review with individual participant data and aggregate data meta-analysis
464 and trial sequential analysis. *Systematic reviews* **3**, 23.

465

466 Giussani DA. (2016). The fetal brain sparing response to hypoxia: physiological mechanisms. *J*
467 *Physiol* **594**, 1215-1230.

468

469 Giussani DA, Camm EJ, Niu Y, Richter HG, Blanco CE, Gottschalk R, Blake EZ, Horder KA, Thakor
470 AS, Hansell JA, Kane AD, Wooding FB, Cross CM & Herrera EA. (2012). Developmental
471 programming of cardiovascular dysfunction by prenatal hypoxia and oxidative stress.
472 *PLoS One* **7**, e31017.

473

474 Giussani DA & Davidge ST. (2013). Developmental programming of cardiovascular disease by
475 prenatal hypoxia. *J Dev Orig Health Dis* **4**, 328-337.

476

477 Gluckman PD, Hanson MA, Cooper C & Thornburg KL. (2008). Effect of in utero and early-life
478 conditions on adult health and disease. *N Engl J Med* **359**, 61-73.

479

480 Grundy D. (2015). Principles and standards for reporting animal experiments in The Journal of
481 Physiology and Experimental Physiology. *J Physiol* **593**, 2547-2549.

482

483 Herraiz S, Pellicer B, Serra V, Cauli O, Cortijo J, Felipe V & Pellicer A. (2012). Sildenafil citrate
484 improves perinatal outcome in fetuses from pre-eclamptic rats. *BJOG* **119**, 1394-1402.

485

486 Herrera EA, Verkerk MM, Derks JB & Giussani DA. (2010). Antioxidant treatment alters
487 peripheral vascular dysfunction induced by postnatal glucocorticoid therapy in rats.
488 *PLoS One* **5**, e9250.

489
490 Itani N, Skeffington KL, Beck C, Niu Y & Giussani DA. (2016). Melatonin rescues cardiovascular
491 dysfunction during hypoxic development in the chick embryo. *J Pineal Res* **60**, 16-26.

492
493 Kane AD, Hansell JA, Herrera EA, Allison BJ, Niu Y, Brain KL, Kaandorp JJ, Derks JB & Giussani
494 DA. (2014). Xanthine oxidase and the fetal cardiovascular defence to hypoxia in late
495 gestation ovine pregnancy. *J Physiol* **592**, 475-489.

496
497 Kane AD, Herrera EA, Camm EJ & Giussani DA. (2013). Vitamin C prevents intrauterine
498 programming of in vivo cardiovascular dysfunction in the rat. *Circ J* **77**, 2604-2611.

499
500 Kass DA, Takimoto E, Nagayama T & Champion HC. (2007). Phosphodiesterase regulation of
501 nitric oxide signaling. *Cardiovasc Res* **75**, 303-314.

502
503 Kissner R, Nauser T, Bugnon P, Lye PG & Koppenol WH. (1997). Formation and properties of
504 peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow
505 technique, and pulse radiolysis. *Chem Res Toxicol* **10**, 1285-1292.

506
507 Koupparis AJ, Jeremy JY, Muzaffar S, Persad R & Shukla N. (2005). Sildenafil inhibits the
508 formation of superoxide and the expression of gp47 NAD[P]H oxidase induced by the
509 thromboxane A2 mimetic, U46619, in corpus cavernosal smooth muscle cells. *BJU Int*
510 **96**, 423-427.

511
512 le Noble FA, Ruijtenbeek K, Gommers S, de Mey JG & Blanco CE. (2000). Contractile and
513 relaxing reactivity in carotid and femoral arteries of chicken embryos. *Am J Physiol*
514 *Heart Circ Physiol* **278**, H1261-1268.

515
516 Lu Z, Xu X, Hu X, Lee S, Traverse JH, Zhu G, Fassett J, Tao Y, Zhang P, dos Remedios C, Pritzker
517 M, Hall JL, Garry DJ & Chen Y. (2010). Oxidative stress regulates left ventricular PDE5
518 expression in the failing heart. *Circulation* **121**, 1474-1483.

519
520 Maiti P, Singh SB, Sharma AK, Muthuraju S, Banerjee PK & Ilavazhagan G. (2006). Hypobaric
521 hypoxia induces oxidative stress in rat brain. *Neurochem Int* **49**, 709-716.

522
523 Marcela SG, Cristina RM, Angel PG, Manuel AM, Sofia DC, Patricia de LR, Bladimir RR &
524 Concepcion SG. (2012). Chronological and morphological study of heart development
525 in the rat. *Anat Rec (Hoboken)* **295**, 1267-1290.

526
527 Masella R, Di Benedetto R, Vari R, Filesi C & Giovannini C. (2005). Novel mechanisms of natural
528 antioxidant compounds in biological systems: involvement of glutathione and
529 glutathione-related enzymes. *J Nutr Biochem* **16**, 577-586.

530
531 Mulder AL, Golde JM, Goor AA, Giussani DA & Blanco CE. (2000). Developmental changes in
532 plasma catecholamine concentrations during normoxia and acute hypoxia in the chick
533 embryo. *J Physiol* **527 Pt 3**, 593-599.

534
535 Nagendran J, Archer SL, Soliman D, Gurtu V, Moudgil R, Haromy A, St Aubin C, Webster L,
536 Rebeyka IM, Ross DB, Light PE, Dyck JR & Michelakis ED. (2007). Phosphodiesterase
537 type 5 is highly expressed in the hypertrophied human right ventricle, and acute
538 inhibition of phosphodiesterase type 5 improves contractility. *Circulation* **116**, 238-
539 248.

540
541 Patterson AJ, Xiao D, Xiong F, Dixon B & Zhang L. (2012). Hypoxia-derived oxidative stress
542 mediates epigenetic repression of PKCepsilon gene in foetal rat hearts. *Cardiovasc Res*
543 **93**, 302-310.

544
545 Pokreisz P, Vandenwijngaert S, Bito V, Van den Bergh A, Lenaerts I, Busch C, Marsboom G,
546 Gheysens O, Vermeersch P, Biesmans L, Liu X, Gillijns H, Pellens M, Van Lommel A,
547 Buys E, Schoonjans L, Vanhaecke J, Verbeken E, Sipido K, Herijgers P, Bloch KD &
548 Janssens SP. (2009). Ventricular phosphodiesterase-5 expression is increased in
549 patients with advanced heart failure and contributes to adverse ventricular remodeling
550 after myocardial infarction in mice. *Circulation* **119**, 408-416.

551
552 Refuerzo JS, Sokol RJ, Aranda JV, Hallak M, Hotra JW, Kruger M & Sorokin Y. (2006). Sildenafil
553 citrate and fetal outcome in pregnant rats. *Fetal Diagn Ther* **21**, 259-263.

554
555 Rouwet EV, Tintu AN, Schellings MW, van Bilsen M, Lutgens E, Hofstra L, Slaaf DW, Ramsay G &
556 Le Noble FA. (2002). Hypoxia induces aortic hypertrophic growth, left ventricular
557 dysfunction, and sympathetic hyperinnervation of peripheral arteries in the chick
558 embryo. *Circulation* **105**, 2791-2796.

559
560 Ruijtenbeek K, De Mey JG & Blanco CE. (2002). The chicken embryo in developmental
561 physiology of the cardiovascular system: a traditional model with new possibilities. *Am*
562 *J Physiol Regul Integr Comp Physiol* **283**, R549-550; author reply R550-541.

563
564 Ruijtenbeek K, le Noble FA, Janssen GM, Kessels CG, Fazzi GE, Blanco CE & De Mey JG. (2000).
565 Chronic hypoxia stimulates periarterial sympathetic nerve development in chicken
566 embryo. *Circulation* **102**, 2892-2897.

567
568 Salinas CE, Blanco CE, Villena M, Camm EJ, Tuckett JD, Weerakkody RA, Kane AD, Shelley AM,
569 Wooding FB, Quy M & Giussani DA. (2010). Cardiac and vascular disease prior to
570 hatching in chick embryos incubated at high altitude. *J Dev Orig Health Dis* **1**, 60-66.

571

572 Samangaya RA, Mires G, Shennan A, Skillern L, Howe D, McLeod A & Baker PN. (2009). A
573 randomised, double-blinded, placebo-controlled study of the phosphodiesterase type
574 5 inhibitor sildenafil for the treatment of preeclampsia. *Hypertens Pregnancy* **28**, 369-
575 382.

576
577 Sanchez-Aparicio P, Mota-Rojas D, Nava-Ocampo AA, Trujillo-Ortega ME, Alfaro-Rodriguez A,
578 Arch E & Alonso-Spilsbury M. (2008). Effects of sildenafil on the fetal growth of guinea
579 pigs and their ability to survive induced intrapartum asphyxia. *Am J Obstet Gynecol*
580 **198**, 127 e121-126.

581
582 Satterfield MC, Bazer FW, Spencer TE & Wu G. (2010). Sildenafil citrate treatment enhances
583 amino acid availability in the conceptus and fetal growth in an ovine model of
584 intrauterine growth restriction. *J Nutr* **140**, 251-258.

585
586 Sharma SK, Lucitti JL, Nordman C, Tinney JP, Tobita K & Keller BB. (2006). Impact of hypoxia on
587 early chick embryo growth and cardiovascular function. *Pediatr Res* **59**, 116-120.

588
589 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK,
590 Goeke NM, Olson BJ & Klenk DC. (1985). Measurement of protein using bicinchoninic
591 acid. *Anal Biochem* **150**, 76-85.

592
593 Spickett CM. (2013). The lipid peroxidation product 4-hydroxy-2-nonenal: Advances in
594 chemistry and analysis. *Redox Biol* **1**, 145-152.

595
596 Stanley JL, Andersson IJ, Poudel R, Rueda-Clausen CF, Sibley CP, Davidge ST & Baker PN. (2012).
597 Sildenafil citrate rescues fetal growth in the catechol-O-methyl transferase knockout
598 mouse model. *Hypertension* **59**, 1021-1028.

599
600 Thakor AS, Herrera EA, Seron-Ferre M & Giussani DA. (2010a). Melatonin and vitamin C
601 increase umbilical blood flow via nitric oxide-dependent mechanisms. *J Pineal Res* **49**,
602 399-406.

603
604 Thakor AS, Richter HG, Kane AD, Dunster C, Kelly FJ, Poston L & Giussani DA. (2010b). Redox
605 modulation of the fetal cardiovascular defence to hypoxaemia. *J Physiol* **588**, 4235-
606 4247.

607
608 Thompson LP & Al-Hasan Y. (2012). Impact of oxidative stress in fetal programming. *J*
609 *Pregnancy* **2012**, 582748.

610
611 von Dadelszen P, Dwinnell S, Magee LA, Carleton BC, Gruslin A, Lee B, Lim KI, Liston RM, Miller
612 SP, Rurak D, Sherlock RL, Skoll MA, Wareing MM, Baker PN, Research into Advanced

613 Fetal D & Therapy G. (2011). Sildenafil citrate therapy for severe early-onset
614 intrauterine growth restriction. *BJOG* **118**, 624-628.

615
616 Walker DK, Ackland MJ, James GC, Muirhead GJ, Rance DJ, Wastall P & Wright PA. (1999).
617 Pharmacokinetics and metabolism of sildenafil in mouse, rat, rabbit, dog and man.
618 *Xenobiotica* **29**, 297-310.

619
620 Welinder C & Ekblad L. (2011). Coomassie Staining as Lading Control in Western Blot Analysis. *J*
621 *Proteome Res* **10**, 1416-1419.

622
623 Wong SL, Leung FP, Lau CW, Au CL, Yung LM, Yao X, Chen ZY, Vanhoutte PM, Gollasch M &
624 Huang Y. (2009). Cyclooxygenase-2-derived prostaglandin F2alpha mediates
625 endothelium-dependent contractions in the aortae of hamsters with increased impact
626 during aging. *Circ Res* **104**, 228-235.

627

628 **ADDITIONAL INFORMATION**

629 None

630 **COMPETING INTERESTS**

631 None

632 **AUTHOR CONTRIBUTIONS**

633 All experiments were performed at The University of Cambridge. Conception and
634 design of experiments and analysis of data: NI, KLS, CB and DAG. Editing of the
635 article: NI, DAG. All authors approve the final version of the manuscript and agree to
636 be accountable for all aspects of the work in ensuring that questions related to the
637 accuracy or integrity of any part of the work are appropriately investigated and resolved.
638 All persons designated as authors qualify for authorship, and all those who qualify for
639 authorship are listed.

640

641 **FUNDING**

642 Supported by the British Heart Foundation. Dino Giussani is the Professor of
643 Cardiovascular Developmental Physiology & Medicine at the Department of
644 Physiology Development & Neuroscience at the University of Cambridge, Professorial

645 Fellow and Director of Studies in Medicine at Gonville & Caius College, a Lister
 646 Institute Fellow and a Royal Society Wolfson Research Merit Award Holder.

647

648 **AUTHORS TRANSLATIONAL PERSPECTIVE**

649 Accumulating data derived from humans and animal models of complicated pregnancy
 650 support potential beneficial effects of sildenafil in improving placental perfusion and
 651 protecting fetal growth. These findings have served as the basis for launching the
 652 STRIDER clinical trials, a large multi-centre international scheme to determine the
 653 efficacy of sildenafil as candidate clinical interventional therapy to improve fetal growth
 654 restriction. However, whether the positive effects of sildenafil transcend those on
 655 placental perfusion and fetal growth onto beneficial effects on the fetal cardiovascular
 656 system was unknown. Equally important, whether sildenafil has any potential adverse
 657 effects on fetal cardiovascular function in healthy or complicated development was
 658 unclear. Here, we show that sildenafil has direct protective effects on the developing
 659 cardiovascular system of the chronically hypoxic fetus. Further, these protective effects
 660 are evident when sildenafil therapy is started long after the onset of chronic fetal
 661 hypoxia. This is useful from a human clinical perspective, as therapy can only be
 662 administered once fetal growth restriction as a result of chronic fetal hypoxia is
 663 diagnosed around 25 weeks of gestation. Therefore, sildenafil may be a good candidate
 664 for human translational antioxidant therapy to protect the chronically hypoxic fetus in
 665 adverse pregnancy.

666 *Word count - 195*

667

TABLE

	N		H		HS		NS		Overall effect of	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Hypoxia	Sildenafil
Heart	0.2	0.01	0.14	0.01 *	0.13	0.01 *	0.17	0	†	P<0.0001
Lung	0.24	0.01	0.14	0.02 *	0.16	0.01 *	0.23	0.01		P<0.0001
Liver	0.57	0.02	0.39	0.02 *	0.42	0.01 *	0.53	0.02		P<0.0001
Kidney	0.23	0.01	0.16	0.02 *	0.18	0.01 *	0.23	0.01		P<0.0001

Heart/BW	0.77	0.02	0.8	0.04	0.66	0.03 †	0.67	0.02 †	P<0.0001
Lung/BW	0.92	0.03	0.84	0.11	0.85	0.06	0.91	0.03	
Liver/BW	2.16	0.08	2.15	0.1	2.21	0.08	2.07	0.04	
Kidney/BW	0.88	0.03	0.89	0.07	0.91	0.05	0.89	0.03	

668

669 **Table 1. Organ weight of chick embryos at day 19 of incubation.** Values are mean
670 and S.E.M at day 19 of absolute (in grams) and relative organ weights (to body weight,
671 BW, in grams/grams) of chick embryos incubated in either N ($n=11$), H ($n=10$), HS
672 ($n=10$) or NS ($n=11$). Significant ($P<0.05$) differences are: * effect of hypoxia (N vs. H
673 and NS vs. HS): † effect of sildenafil (N vs. NS and H vs. HS). Two-way ANOVA.
674 There was no interaction found between the effect of hypoxia and of sildenafil treatment.

675

FIGURES

676

677 **Figure 1. Haematocrit and fetal biometry.** Values are mean \pm S.E.M at day 19 of
678 haematocrit (A), embryo weight (B), brain weight relative to body weight (C) and
679 resource partitioning (D) of chick embryos incubated in either normoxia (N, $n=11$),
680 hypoxia (H, $n=10$), hypoxia with sildenafil (HS, $n=10$) or normoxia with sildenafil (NS,
681 $n=11$). Significant ($P<0.05$) differences are: * effect of hypoxia (N vs. H and NS vs.
682 HS). Two-way ANOVA. There was no interaction found between the effect of hypoxia
683 and of sildenafil treatment.

684 **Figure 2. Pro-oxidant mechanisms.** Values are mean \pm S.E.M. at day 19 of the
685 expression of 3-NT (A) and 4-HNE (B) in the heart of chick embryos incubated in
686 either N, H, HS or NS. $n = 8, 8, 9, 9$, respectively. Significant ($P<0.05$) differences are:
687 * effect of hypoxia (N vs. H and NS vs. HS): † effect of sildenafil (N vs. NS and H vs.
688 HS). Two-way ANOVA with no interaction (A) or with interaction and Bonferroni *post*
689 *hoc* test (B).

690 **Figure 3. Anti-oxidant mechanisms and NO bioavailability.** Values are mean \pm
691 S.E.M. at day 19 of the expression of SOD (A), the activity of catalase (B), the
692 expression of GPx (C), and the concentration of NOx (D) in the heart of chick embryos

693 incubated in either N, H, HS or NS. n = 9, 9, 8, 8 for A and B. n = 6 for all groups for C.
694 n = 8, 8, 9, 9, respectively, for D. Significant ($P < 0.05$) differences are: * effect of
695 hypoxia (N vs. H and NS vs. HS): † effect of sildenafil (N vs. NS and H vs. HS). Two-
696 way ANOVA with no interaction (A, B and C) or with interaction and Bonferroni *post*
697 *hoc* test (D).

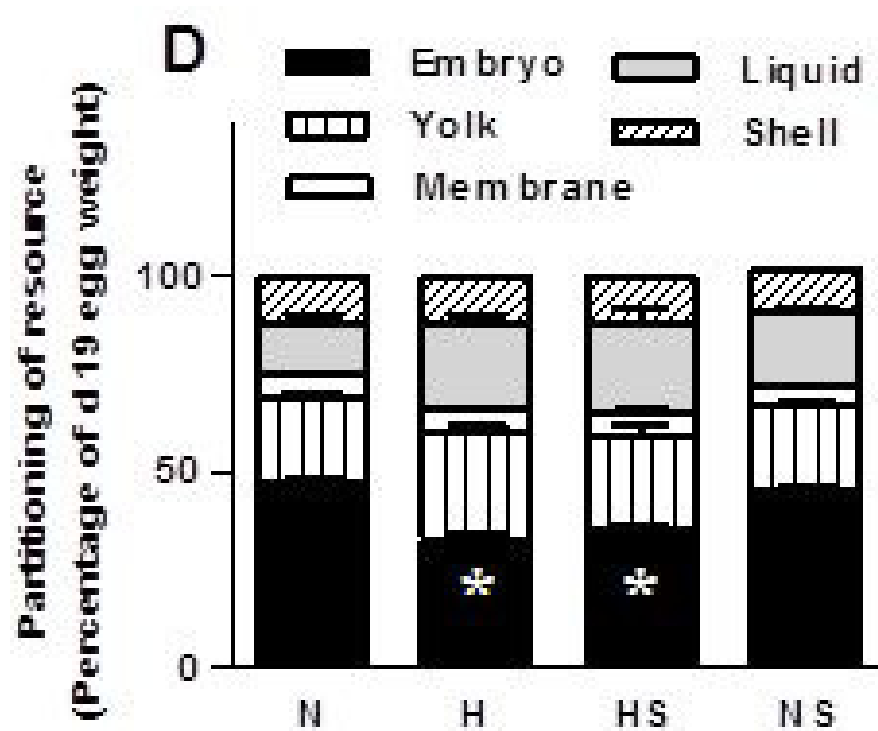
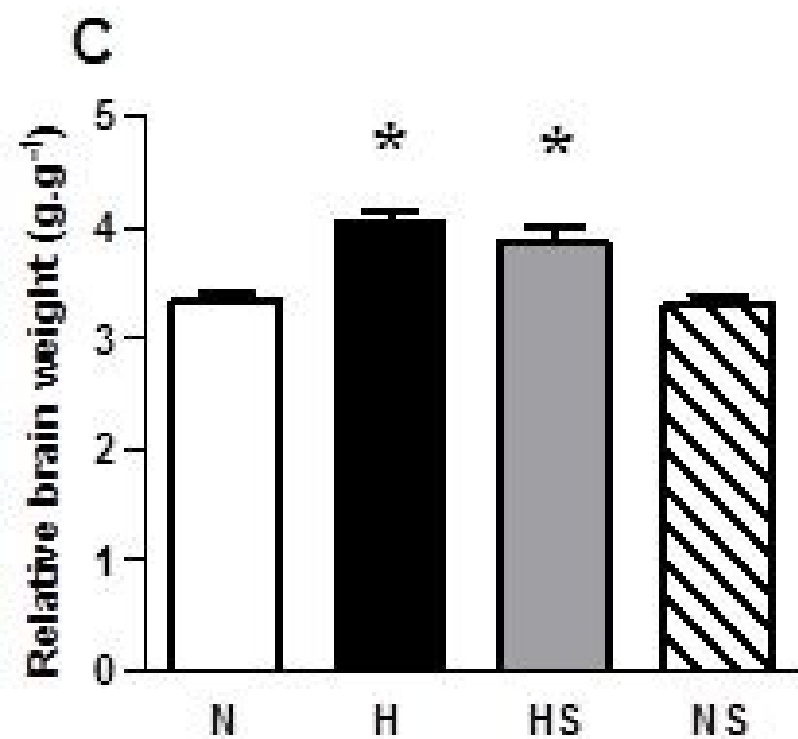
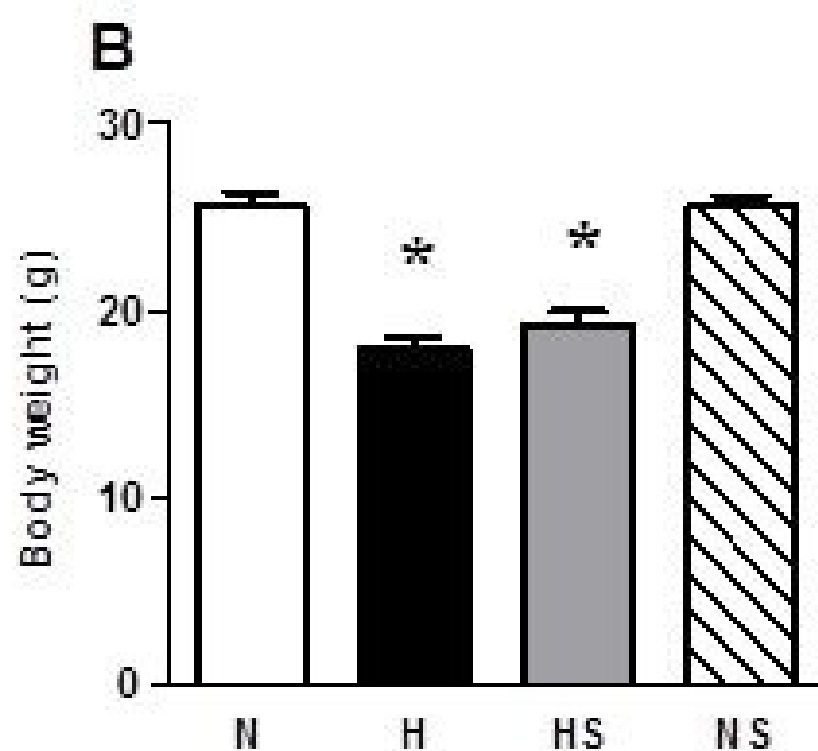
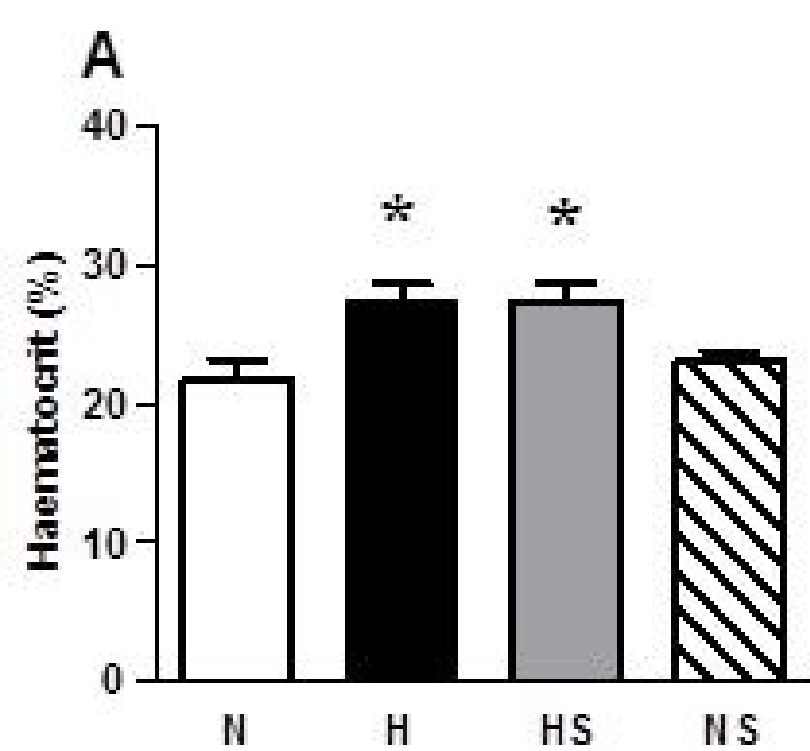
698 **Figure 4. Cardiac PDE5 expression.** Values are mean \pm S.E.M. at day 19 for the
699 expression of PDE5 protein in the heart of chick embryos incubated in either N, H, HS
700 or NS. n = 6 for all groups. Significant ($P < 0.05$) differences are: * effect of hypoxia (N
701 vs. H): † effect of sildenafil (H vs. HS). Two-way ANOVA with interaction and
702 Bonferroni *post hoc* test.

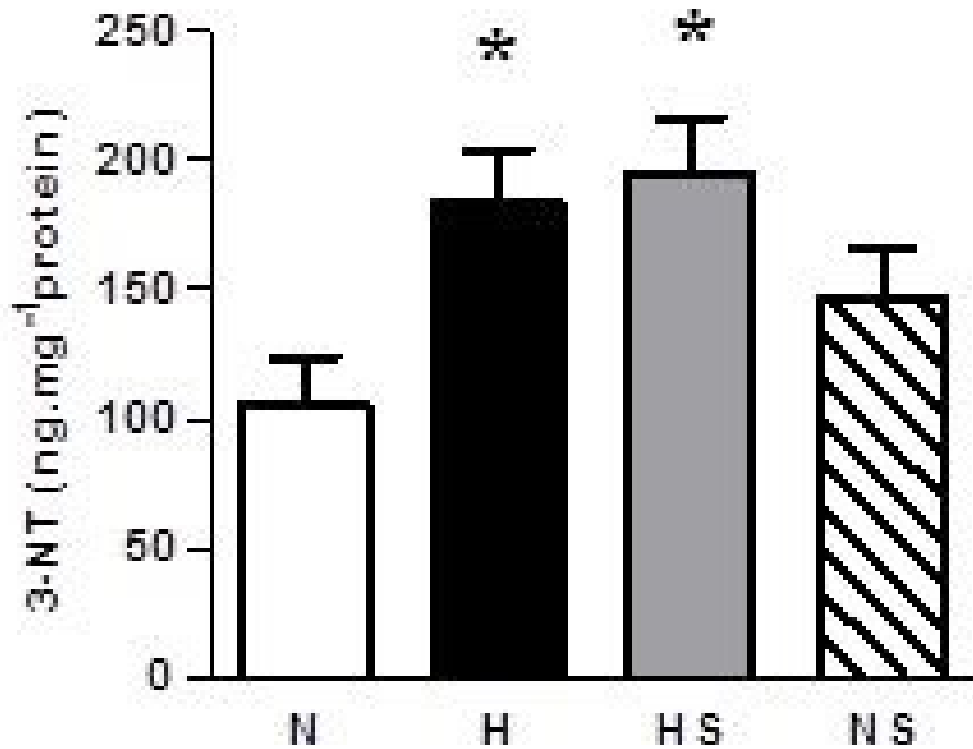
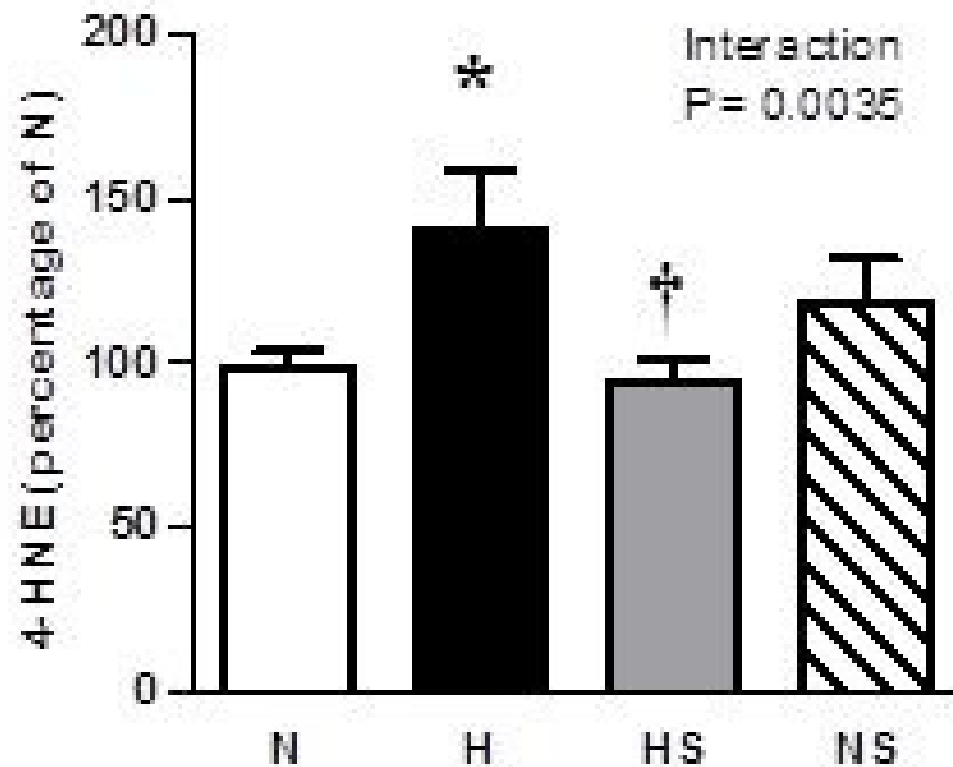
703

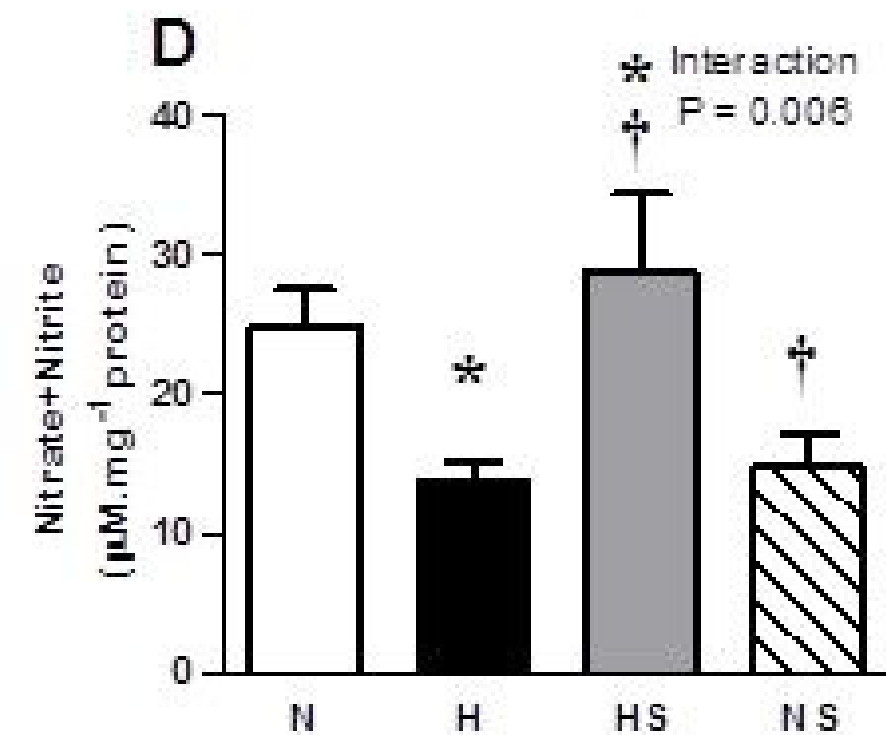
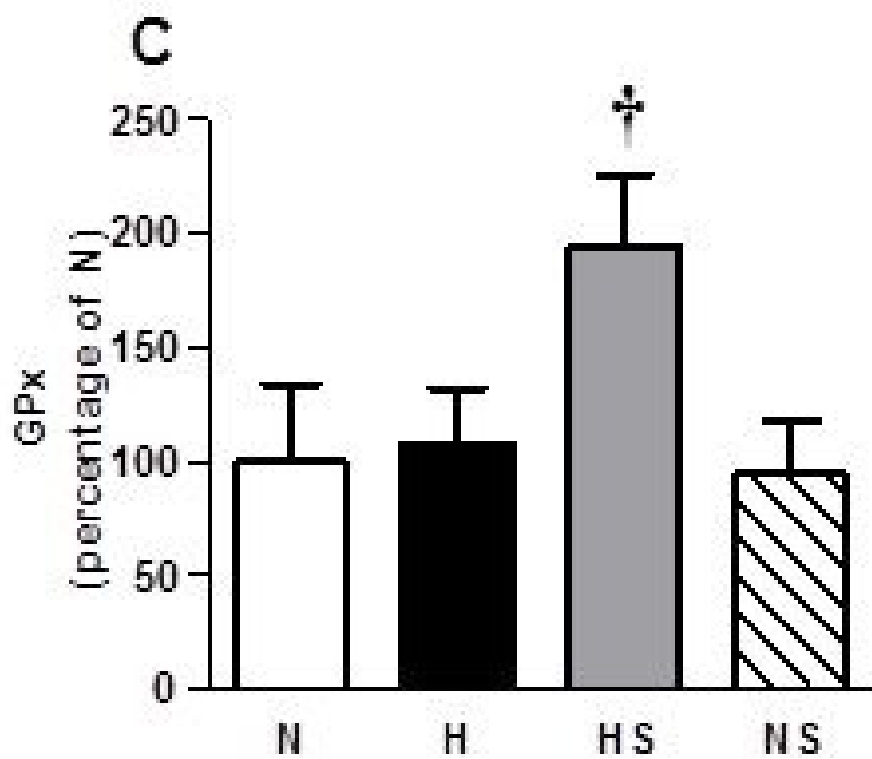
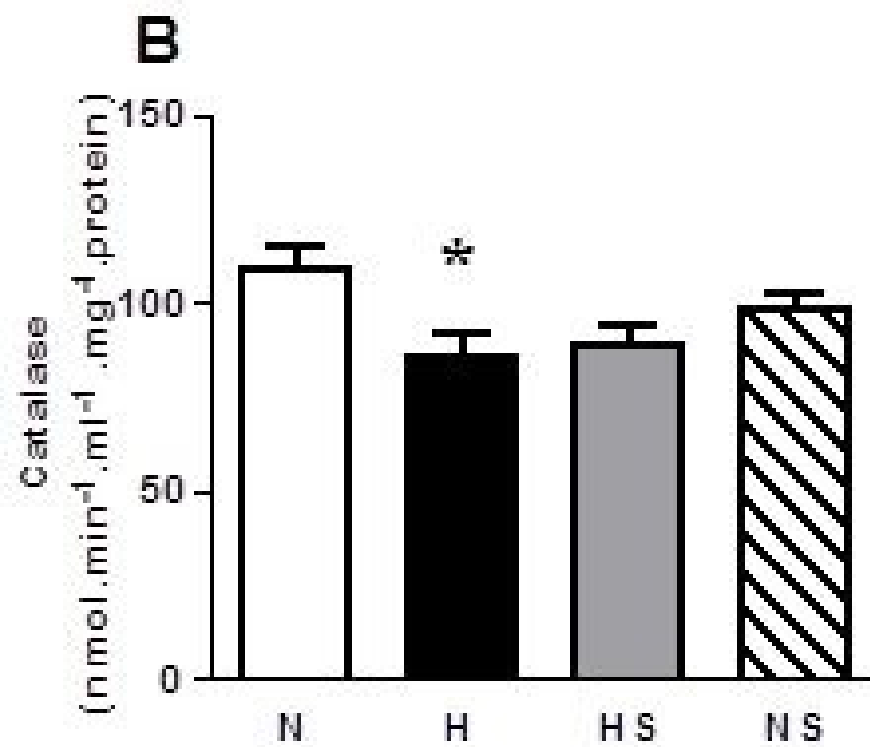
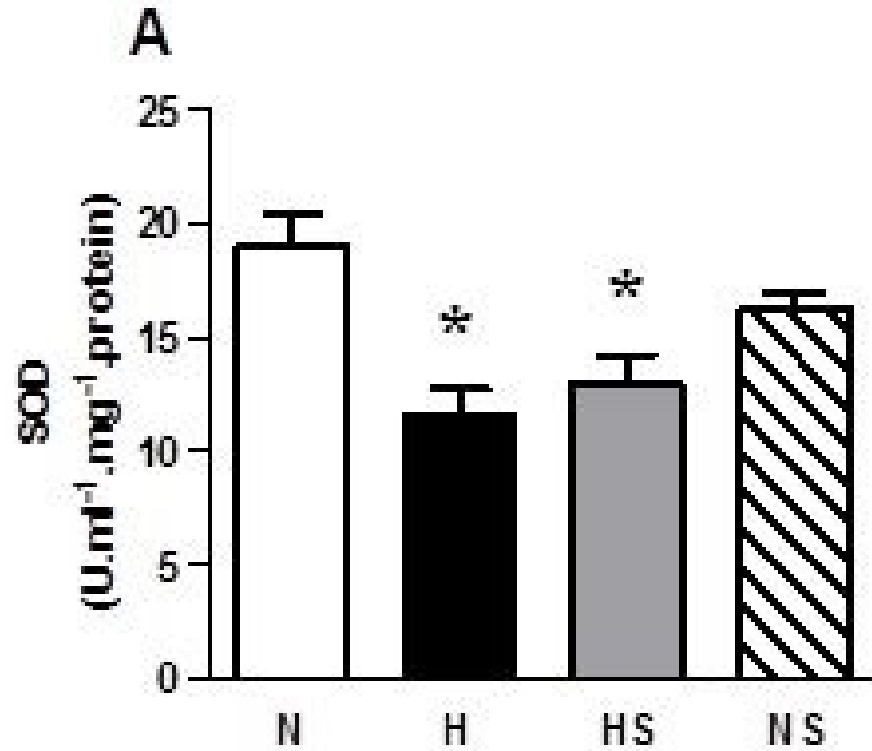
704 **Figure 5.** Representative recording of the acetylcholine dose-response curves. Example
705 recordings of a femoral arterial segment of 2mm that was exposed to cumulative doses
706 of acetylcholine (ACh) isolated from chick embryos incubated in either N, H, HS or NS.
707 The traces are shown as time (minutes, horizontal axis) vs. vascular wall tension
708 (mN/mm, vertical axis). The ACh doses were given at two minute intervals.
709 Concentration of ACh are shown as $-\log_{10}$ M.

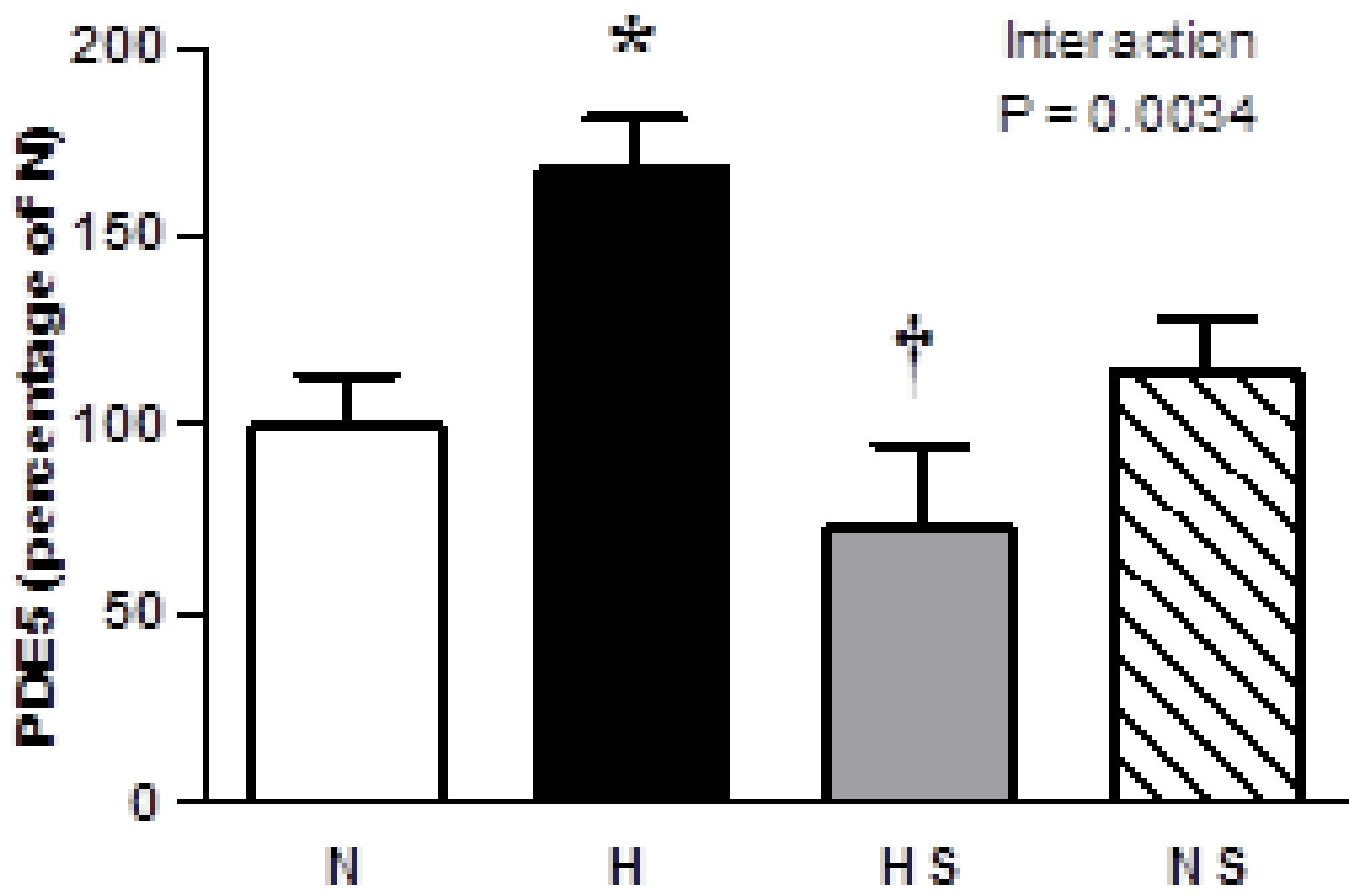
710

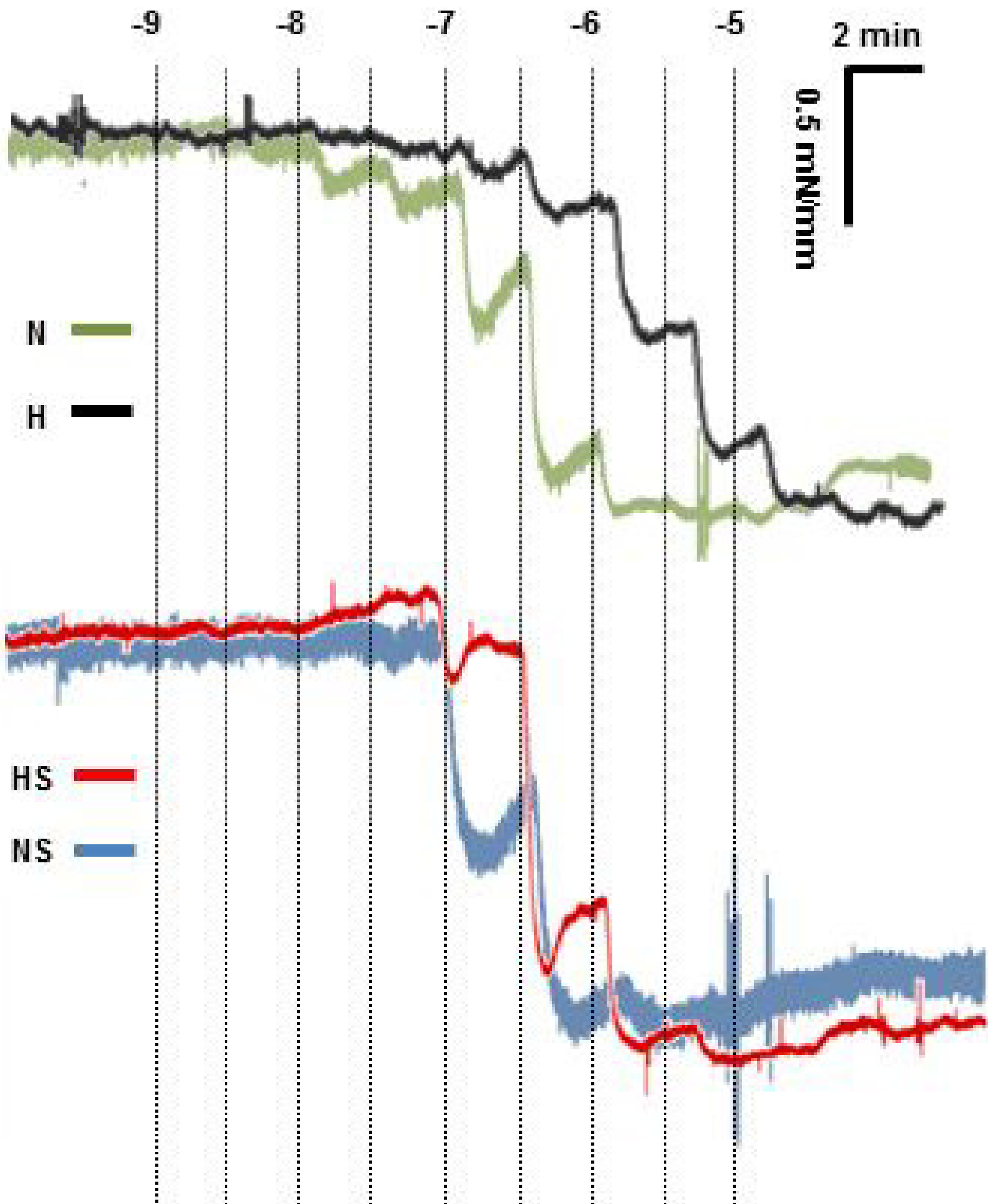
711 **Figure 6. Peripheral vasodilator function.** Values are mean \pm S.E.M. for relaxant
712 responses to SNP (A) and to ACh (B) and vasodilatation to ACh expressed as area
713 under the curve before and after L-NAME treatment (AUC, C) for femoral arterial
714 segments isolated from chick embryos incubated in either N, H, HS and NS. n = 10 for
715 all groups. In (C) the AUC represents ACh-induced relaxation (complete bar with
716 positive S.E.M.), for ACh-induced relaxation following treatment with L-NAME (NO-
717 independent component, grey bar with negative S.E.M.), and for the remaining AUC
718 after ACh with L-NAME (NO-dependent component, black bar with negative white
719 S.E.M.). Significant ($P < 0.05$) differences are: * effect of hypoxia (N vs. H): † effect of
720 sildenafil (H vs. HS) for pD2 (B) and AUC (C) . Two-way ANOVA with interaction
721 and Bonferroni *post hoc* test.

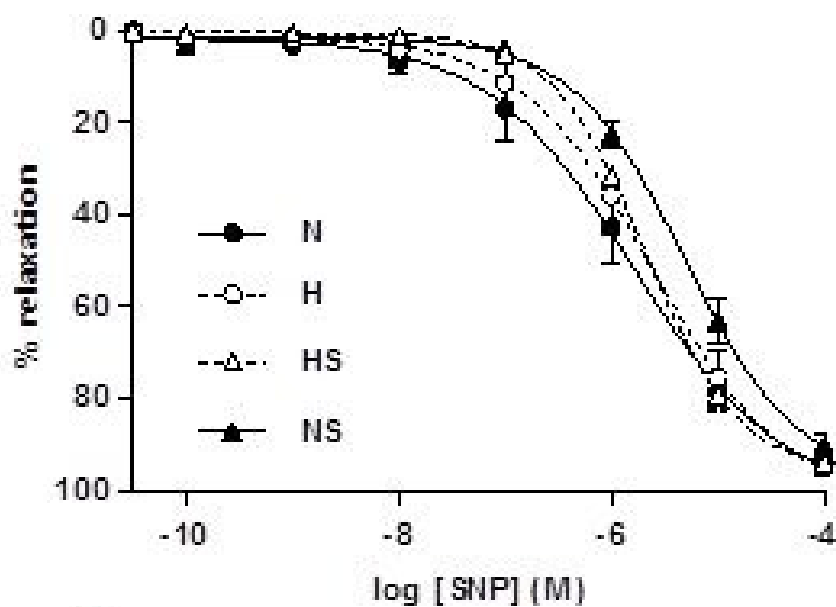
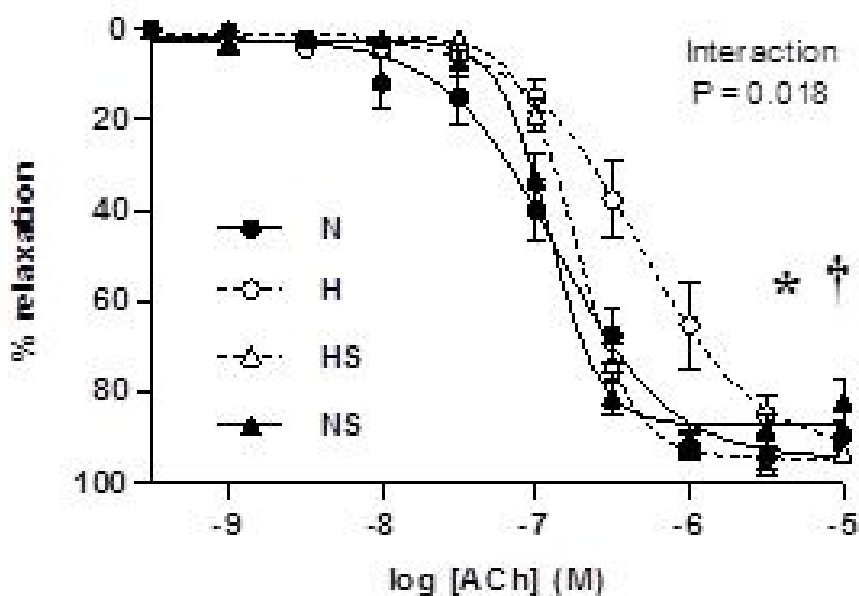


A**B**







A**B****C**