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

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

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KEY POINTS SUMMARY

- 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.

ABSTRACT

There is a need for developing clinically translatable therapy for preventing fetal origins of cardiovascular disease in pregnancy complicated by chronic fetal hypoxia. Evidence shows that sildenafil protects placental perfusion and fetal growth. However, whether beneficial effects of sildenafil transcend onto the fetal heart and circulation in complicated development is unknown. We isolated the direct effects of sildenafil on the fetus using the chick embryo and hypothesised that sildenafil also protects fetal cardiovascular function in hypoxic development. Chick embryos (n=11 per group) were incubated in normoxia or hypoxia (14% O_2) from day 1 and treated with sildenafil (4mg/kg/day) from day 13 of the 21-day incubation. Hypoxic incubation increased oxidative stress (4-hydroxynoneal, 141.1 ± 17.6% of normoxic control), reduced superoxide dismutase (60.7 ± 6.3%), increased phosphodiesterase type 5 expression (167 ± 13.7%) and decreased nitric oxide bioavailability (54.7 ± 6.1%) in the fetal heart, and promoted peripheral endothelial dysfunction (70.9 ± 5.6 AUC of normoxic control; all $P < 0.05$). Sildenafil treatment after onset of chronic hypoxia prevented the increase in phosphodiesterase expression (72.5 ± 22.4), protected against oxidative stress (94.7 ± 6.2) and normalised nitric oxide bioavailability (115.6 ± 22.3) in the fetal heart, and restored endothelial function in the peripheral circulation (89.8 ± 2.9). Sildenafil protects the fetal heart and circulation directly in hypoxic development via mechanisms including decreased oxidative stress and enhanced nitric oxide bioavailability. Sildenafil may be a good translational candidate for human antioxidant therapy to prevent fetal origins of cardiovascular dysfunction in adverse pregnancy.

Abbreviations

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

INTRODUCTION

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

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

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

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

METHODS

Ethical Approval

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

Animals

Fertilised Bovans Brown eggs (Medeggs, Norfolk, UK) were weighed and incubated under normoxic (21% O_2) or hypoxic (14±0.5% O_2) conditions (37.9°C, 45% humidity, 12:12h light:dark cycle, automatic rotation every hour, Mod-75A equipped with electronic servo-controlled humidity system HS-Auto-3.5L, Marsalles, Barcelona, Spain) from day 1. The levels of oxygen, humidity and temperature inside the incubators were continuously monitored (DD103 DrDAQ Oxygen Sensor, Pico Technology, St. Neots, UK).

Dose of sildenafil

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

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

Haematocrit and growth analysis

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

Molecular studies in the chick embryo heart

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

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

The cardiac expression of GPx and PDE5 was determined by Western Blot. Frozen chick hearts (25mg) were powdered on dry ice and homogenized in 250µl of ice-cold lysis buffer (HEPES:50mM, NaCl:150mM, Triton-X100:1%, Na_3VO_4:1mM, NaF:30mM, $Na_4P_2O_7$:10mM, EDTA:10mM, Protease inhibitor cocktail III [Calbiochem, Nottingham, UK]) in a microtube containing 1.4 mm ceramic beads (Lysing Matrix D, MP biomedicals). The protein concentration of lysates was determined using the copper-Bicinchoninic assay (Smith *et al.*, 1985). Protein samples were diluted with 5x Laemmli's buffer (sodium dodecyl sulphate (SDS):2%, Tris-HCl (pH 6.8):62.5mM, glycerol:10%, dithiothreitol:100mM, bromophenol blue) then standardized to a protein concentration of 1 mg/ml by further addition of 1 x Laemmli's buffer. Total protein (10 µg) from each sample (n = 6 per treatment group) was separated on SDS-PAGE gel electrophoresis along with a pre-stained molecular weight marker (PageRuler Plus Prestained Protein Ladder 10-250kDa, Thermo Scientific, Waltham, USA), then transferred immediately to a Polyvinylidene difluoride Immobilon-P membrane (PVDF, Millipore, Billerica, MA, USA). The membrane was incubated in blocking buffer before incubation in primary antibodies (GPx, ab22604; PDE5, ab64179; Abcam) overnight at 4°C. Following primary antibody incubation the membranes were incubated with the secondary antibody (Peroxidase-AffiniPure Donkey Anti-Rabbit IgG (H+L), 1:10000 in PBS with 1% marvel and 0.1% Tween 20, Jackson ImmunoResearch laboratories, PA, USA) and immunoreactivity was measured using West Pico chemiluminescent substrate (Thermo Scientific). The intensity of the bands were analysed with the AlphaEase imaging software (Alpha Innotech, San Leandro, CA, USA). Following the protein detection, the membrane was stained with 0.1% Coomassie R-250 and the intensity of the Coomassie staining for each lane was analysed (Welinder & Ekblad, 2011). The expression level of the target protein in each sample was normalised to the Coomassie staining of the same sample for a loading control.

Functional peripheral vascular reactivity using *in vitro* wire myography

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

Denmark) as previously described (Itani *et al.*, 2016). Briefly, a third order femoral artery was dissected at day 19 of incubation and mounted in a chamber containing Kreb's buffer. Vascular constrictor capacity was assessed with increasing doses of K^+ solutions (16.74 – 250 mM) and of phenylephrine (PE, 10^{-8} – 10^{-4} M). The response to K^+ was normalised to the diameter of the vessel (mN/mm/µm/1000). The response to phenylephrine was normalised to the constrictor response to 125mM K^+ achieved by the same vessel (% K^+125). Vasodilator responses to cumulative doses of sodium nitroprusside (SNP, 10^{-10} – 10^{-4} M) and of acetylcholine (ACh, 10^{-9} – 10^{-5} M) were assessed after pre-constricting the vessel with a sub-optimal dose of potassium. The partial contributions of endogenous NO-dependent and NO-independent mechanisms to the vasorelaxation were determined by repeating the ACh dose response curve after incubating the vessel with L-NAME (10^{-5} M, 10min) and calculating the area under the curves (Herrera *et al.*, 2010; Itani *et al.*, 2016). The sensitivity (pD2) to ACh was defined as $-\log_{10}$ (EC50). LabChart was used for data acquisition and analysis of the *in vitro* wire myography data (LabChart 6.0, Powerlab 8/30; AD Instruments, Chalgrove, UK).

Statistical analysis

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

RESULTS

Haematocrit and fetal growth

Incubation under hypoxic conditions from day 1 significantly increased haematocrit in the chick embryo by day 19 (Figure 1A). Exposure to hypoxia throughout development reduced the body weight (Figure 1B) which persisted when the body weight was normalised to the egg weight at the start of incubation (N: 41.3±1.0, H: 29.0±1.3*, HS: 32.0±2.2*, NS: 43.9±1.3 g/g, $P < 0.05$; *effect of hypoxia). Hypoxia affected the body weight more severely than the body length (N: 68.1±1.7, H: 63.2±1.0*, HS: 65.9±0.9, NS: 70.8±0.6 mm, $P < 0.05$; *versus N) of the embryo. Consequently, hypoxic embryos had a lower BMI (N: 5.3±0.2, H: 4.6±0.1*, HS: 4.5±0.2*, NS: 5.1±0.1 kg/m^2, $P < 0.05$; *effect of hypoxia). The brain weight was reduced in the hypoxic embryo (N: 0.84±0.01, H: 0.73±0.02*, HS: 0.74±0.01*, NS: 0.84±0.01 g, $P < 0.05$; *effect of hypoxia), however this was not proportional to the reduction in their body size and thus, the relative brain weight was increased (Figure 1C). In addition, calculation of resource partitioning revealed less resource attributed to embryonic mass in hypoxic incubations (Figure 1D). Collectively, the data show that those embryos exposed to chronic hypoxia were thin for their length and had relative brain sparing. Sildenafil treatment from day 13 of incubation had no effect on changes in fetal growth or brain sparing during either hypoxic or normoxic incubation (Figure 1).

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

Molecular studies in the chick embryo heart

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

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

Functional peripheral vascular reactivity using *in vitro* wire myography

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

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

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

DISCUSSION

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

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

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

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

Delannoy *et al.*, 2010; Giussani, 2016). In the present study, treatment with sildenafil of hypoxic chick embryos restored the endothelium-dependent relaxation of the femoral artery by enhancing NO-dependent mechanisms. This agrees with the principal dilator mechanism of action of sildenafil, enhancing downstream signalling of NO. The ratio of $\bullet O_2^-$:NO yields a vascular oxidant tone and we have shown that this is functional in fetal life and that it can be manipulated in favour of dilation (Thakor *et al.*, 2010a; Thakor *et al.*, 2010b; Giussani *et al.*, 2012; Kane *et al.*, 2014). Mechanisms in addition to antioxidant properties underlying the beneficial effects of sildenafil in the chronically hypoxic fetus therefore include inhibition of the degradation of cyclic GMP by PDE5, enhancing the action of NO, thereby normalising the vascular oxidant tone and endothelial function.

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

In the present study, treatment with sildenafil of hypoxic chick embryos did not improve the fetal growth restriction but brain sparing in growth-restricted fetuses was preserved. Alterations in the ratio of $\bullet O_2^-$:NO promoting a vascular oxidant tone may have a significant effect on circulations which are particularly sensitive to NO, such as the placental and umbilical vascular bed (Derks *et al.*, 2010; Thakor *et al.*, 2010a). In support, a number of studies in humans and mammalian animal models have reported

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

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

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ADDITIONAL INFORMATION

None

COMPETING INTERESTS

None

AUTHOR CONTRIBUTIONS

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

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AUTHORS TRANSLATIONAL PERSPECTIVE

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

Word count - 195

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	

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

FIGURES

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

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

Figure 3. Anti-oxidant mechanisms and NO bioavailability. Values are mean ± S.E.M. at day 19 of the expression of SOD (A), the activity of catalase (B), the 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).

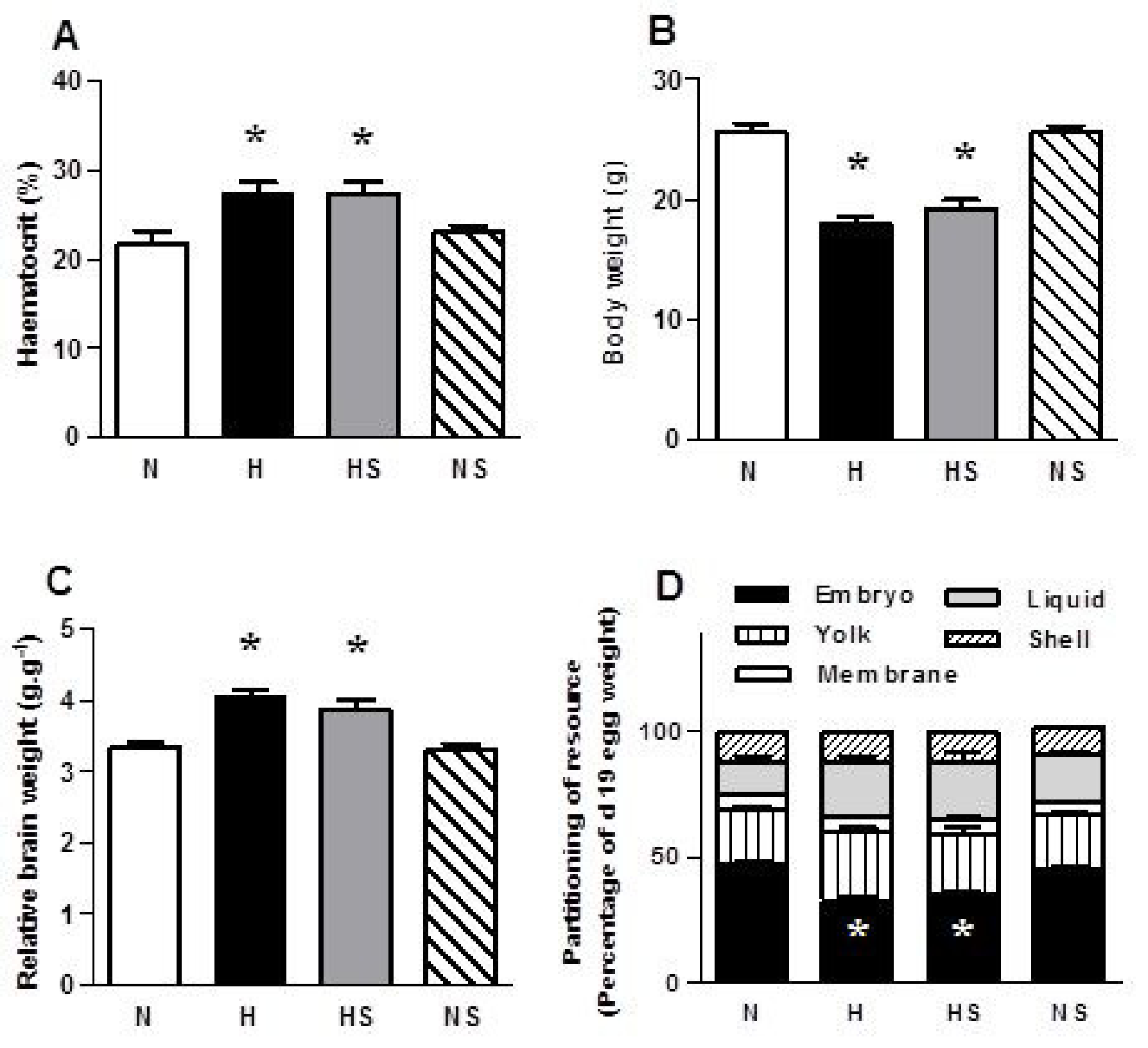
698 **Figure 4. Cardiac PDE5 expression.** Values are mean ± S.E.M. at day 19 for the
699 expression of PDE5 protein in the heart of chick embryos incubated in either N, H, HS
700 or NS. n = 6 for all groups. Significant (*P<0.05*) differences are: * effect of hypoxia (N
701 *vs*. H): † effect of sildenafil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

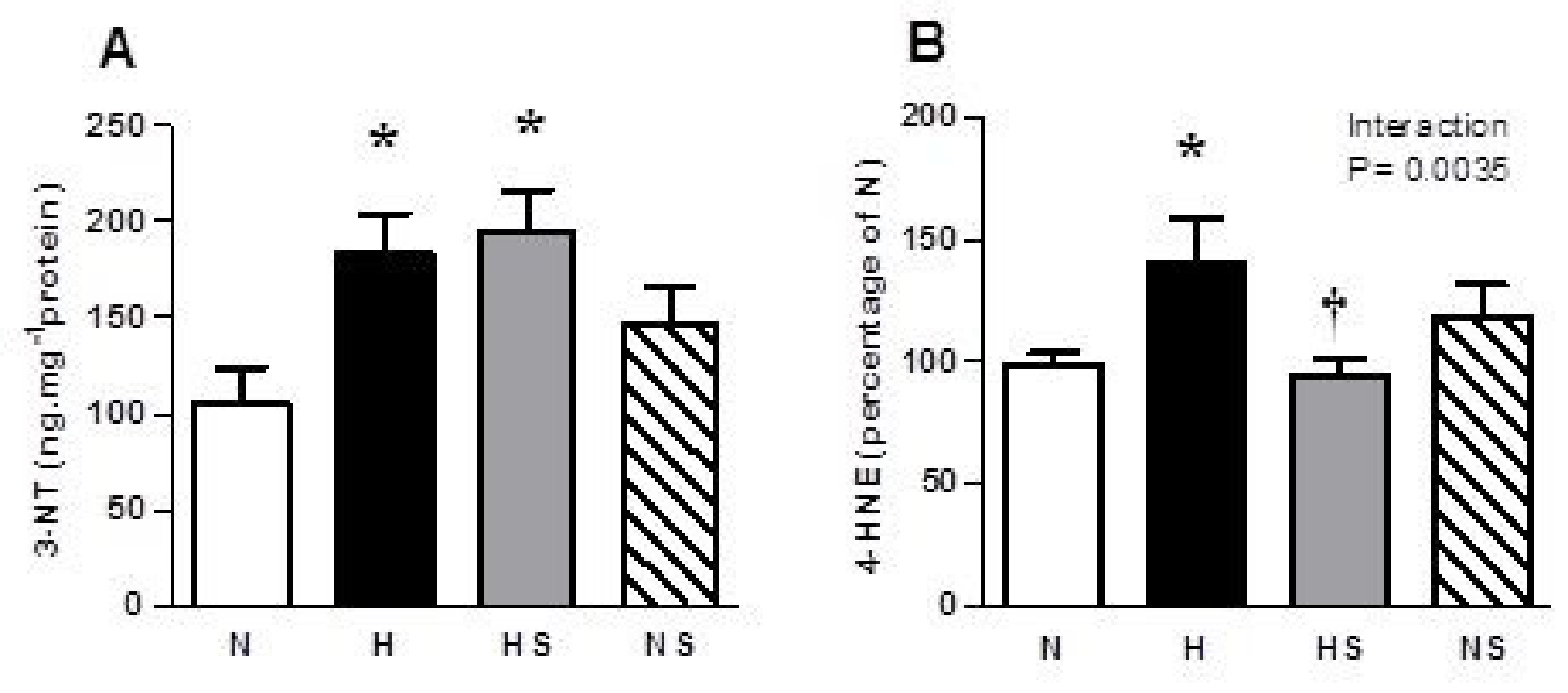
703

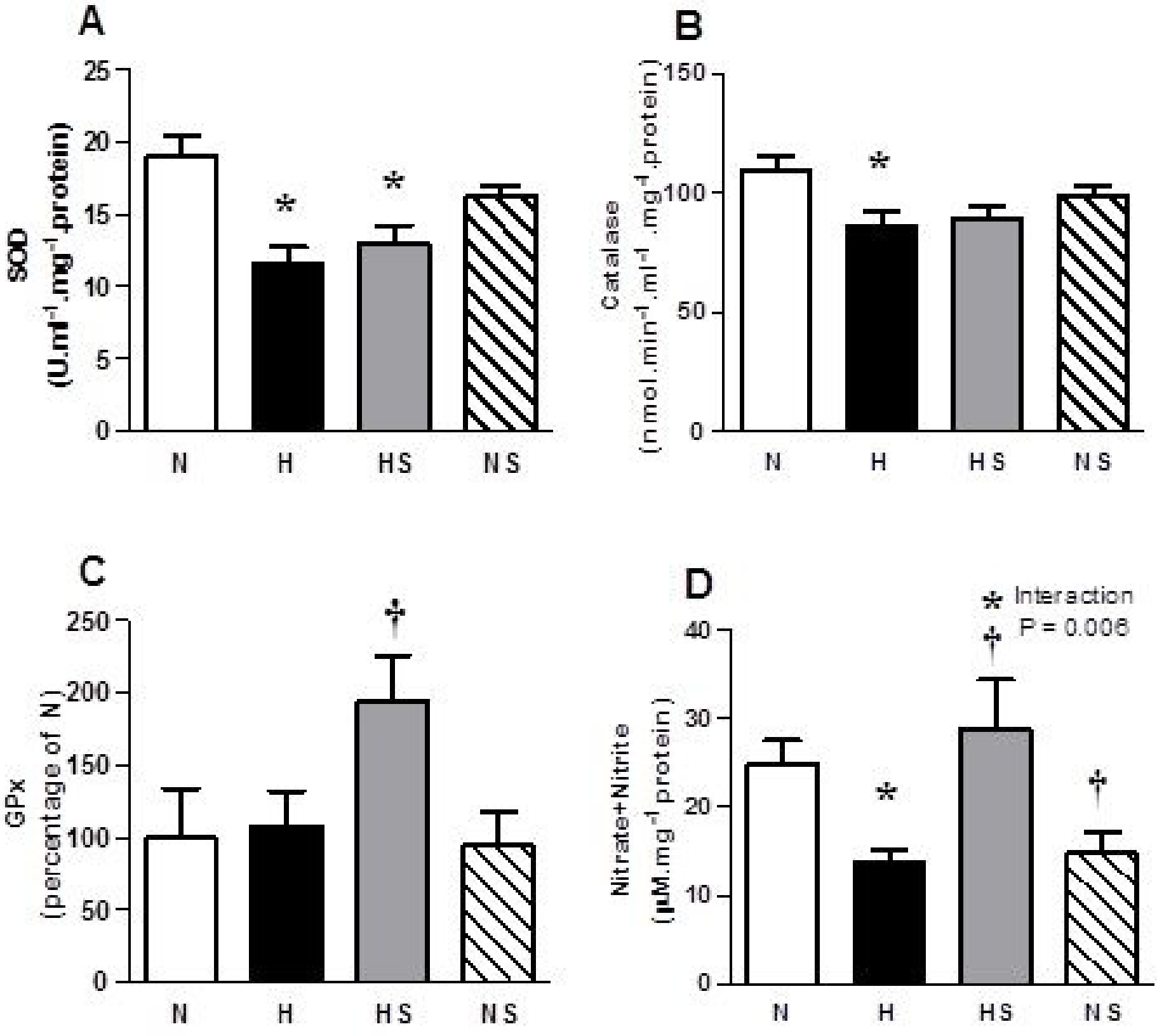
704 **Figure 5.** Representative recording of the acetylcholine dose-response curves. Example
705 recordings of a femoral arterial segment of 2mm that was exposed to cumulative doses
706 of acetylcholine (ACh) isolated from chick embryos incubated in either N, H, HS or NS.
707 The traces are shown as 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.

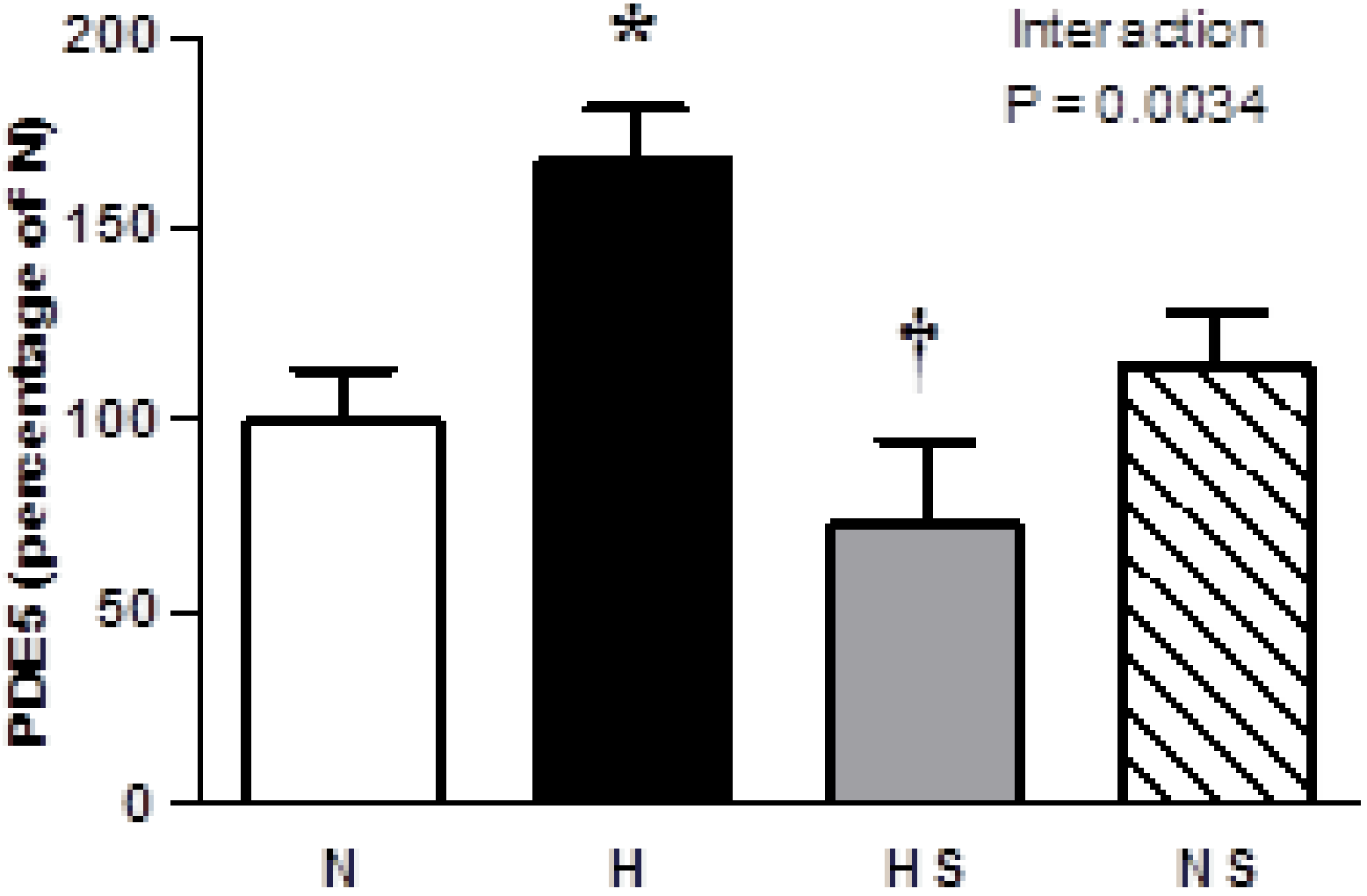
710

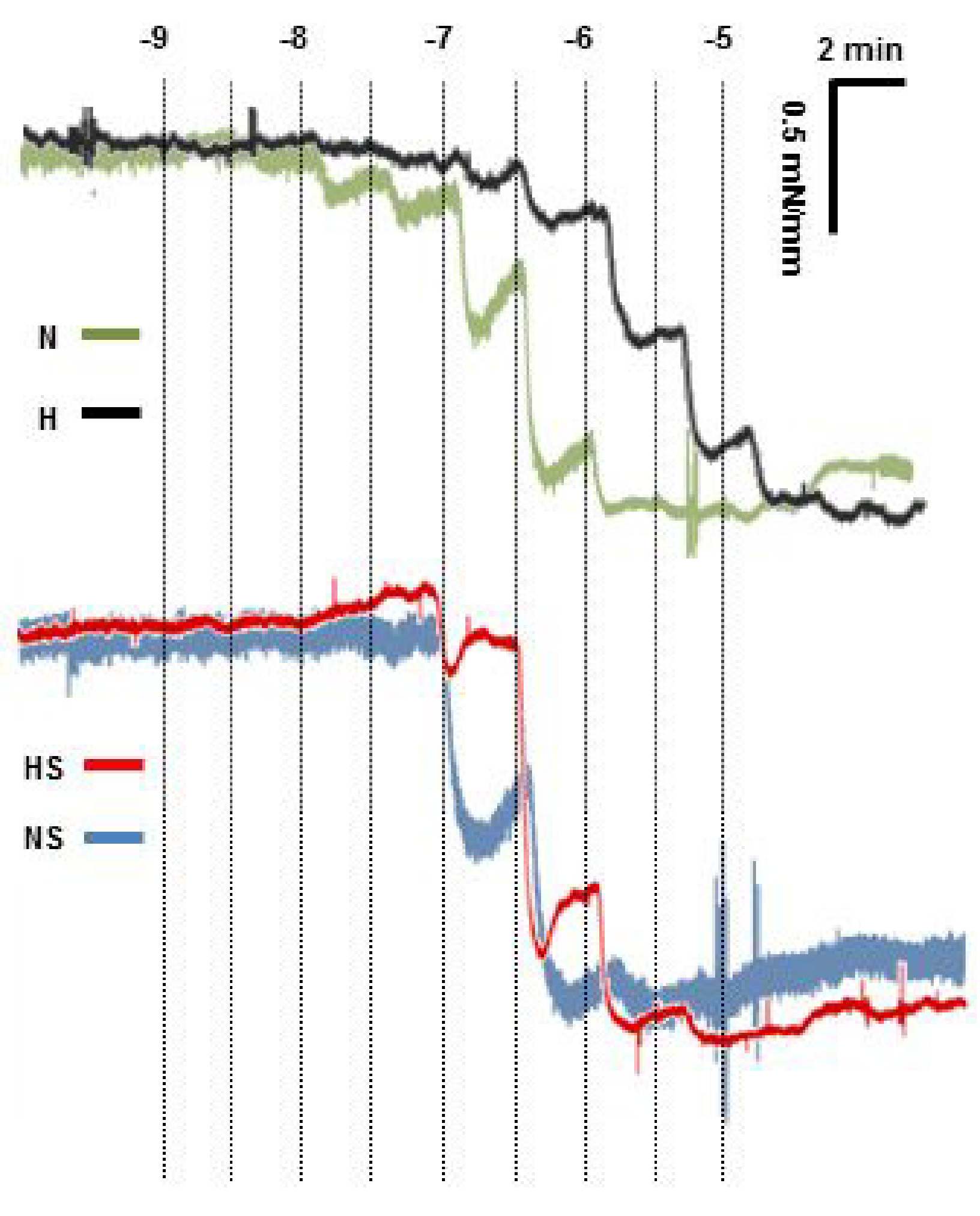
711 **Figure 6. Peripheral vasodilator function.** Values are mean ± S.E.M. for relaxant
712 responses to SNP (A) and to ACh (B) and vasodilatation to ACh expressed as area
713 under the curve before and after L-NAME treatment (AUC, C) for femoral arterial
714 segments isolated from chick embryos incubated 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.

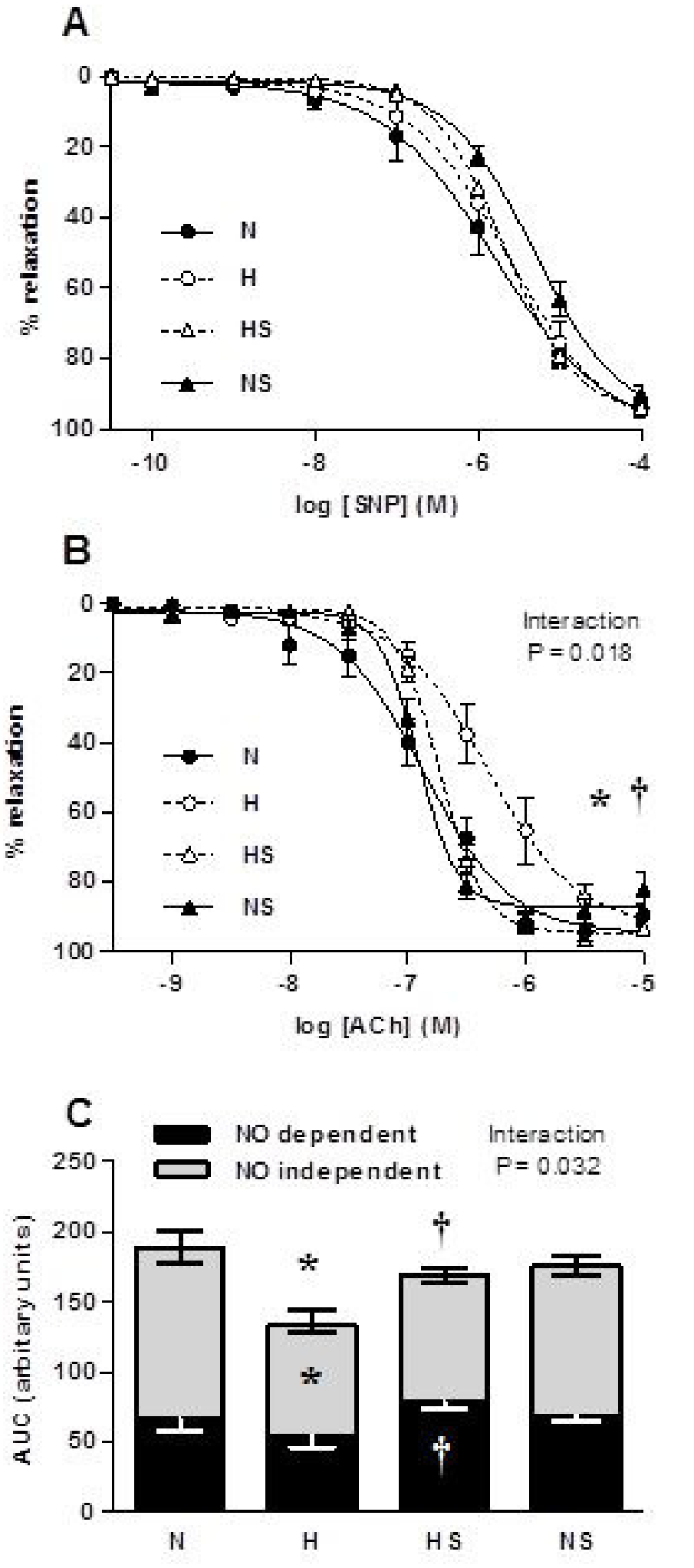

A
Haemtocrit (%)
40
30
20
10
0
*
*
N
H
HS
NS
B
Body weight (g)
30
20
10
0
*
*
N
H
HS
NS
C
Relative brain weight (g.g-1)
5
4
3
2
1
0
*
*
N
H
HS
NS
D
Partitioning of resource
(Percentage of d 19 egg weight)
Embryo
Yolk
Membrane
Liquid
Shell
100
50
0
*
*
N
H
HS
NS


A
250
200
150
100
50
0
3-NT (ng.mg-1protein)
*
*
N
H
HS
NS
B
200
150
100
50
0
4-HNE (percentage of N)
*
†
Interaction
P= 0.0035
N
H
HS
NS


A
SOD
(U.ml-1.mg-1protein)
25
20
15
10
5
0
*
*
N
H
HS
NS
B
Catalase
(n mol.min-1.ml-1 .mg-1.protein)
150
100
50
0
*
N
H
HS
NS
C
GPx
(percentage of N)
250
200
150
100
50
0
†
N
H
HS
NS
D
Nitrate+Nitrite
(µM.mg-1protein)
40
30
20
10
0
* Interaction
† P = 0.006
*
†
†
N
H
HS
NS


200
150
100
50
0
PDE5 (percentage of N)
*
†
Interaction
P = 0.0034
N
H
H S
N S


-9
-8
-7
-6
-5
2 min
0.5 mN/mm
N
H
HS
NS


A
0
20
40
60
80
100
% relaxation
N
H
HS
NS
-10
-8
-6
-4
log [SNP] (M)
B
0
20
40
60
80
100
% relaxation
Interaction
P = 0.018
N
H
HS
NS
*
†
-9
-8
-7
-6
-5
log [ACh] (M)
C
NO dependent
NO independent
Interaction
P = 0.032
250
200
150
100
50
0
AUC (arbitary units)
*
*
†
†
N
H
HS
NS