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Brain apoptosis and carotid artery reactivity in fetal asphyctic preconditioning

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1. ABSTRACT

We aimed to develop a model of fetal hypoxia-ischemia (HI) preconditioning that reflects the pathophysiological conditions of perinatal asphyxia more closely than the existing neonatal stroke models. Fetal asphyxia (FA) was induced by clamping the uterine vasculature on embryonic day E17. At birth (P0), severe perinatal asphyxia (SPA) was induced during cesarean section. At P4, carotid arteries were studied in a wire myograph and at P8 brains were analyzed for apoptotic cell death in the prefrontal cortex and striatum. The contraction induced by K+ was significantly reduced in the carotid arteries from the SPA group and endothelium-dependent relaxation (mediated by acetylcholine) was augmented in the FA group. These changes in vascular responsiveness were not present in the animals exposed to both insults (FA + SPA). Additionally, FA+SPA animals showed lower numbers of apoptotic cells compared to SPA animals in both the prefrontal cortex and striatum. Exposure to a global fetal asphyctic insult seems to protect against the vascular alterations and the increase of apoptosis in striatum and prefrontal cortex induced by severe asphyxia at birth.

2. INTRODUCTION

Developmental asphyxia is a major cause of neonatal mortality and morbidity, which is frequently associated with long-term motor, behavioral and cognitive deficits in survivors (1-6). Although our understanding has increased considerably during the last decade, there is still a lack of effective therapeutic strategies against asphyctic brain damage. Interestingly, brief episodes of hypoxia-ischemia (HI) in the brain can provoke an endogenous neuroprotection against subsequent more severe episodes. This phenomenon is called "hypoxic-ischemic preconditioning" or "hypoxic-ischemic tolerance". Little is known about the exact mechanisms underlying this phenomenon. A better understanding of these mechanisms of neuroprotection can offer interesting targets for the development of new therapeutic approaches.

Currently, various animal models are being used to study HI preconditioning in the brain. Most studies have used adult stroke models, combining different types of artery occlusion (i.e. common carotid artery and middle cerebral artery) and exposure to low oxygen concentrations (7-10). There are, however, fewer models to study

preconditioning in the immature brain. Gidday *et al.* reported that exposure of 7-day-old pups to 8% oxygen induced a robust neuroprotection against a stroke (combination of FiO2 of 8% and carotid occlusion) one day later (11). Another model used is based on clamping of the uterine circulation at embryonic day 17 (E17) followed stroke at postnatal day 7 (P7) (12). Therefore, almost all recent preconditioning models in the immature brain aim to influence or modulate the effects of a neonatal stroke (i.e. focal insult). Focal insults, however, differ from the more relevant clinical situation at birth because they lack multi-organ injury, which is present in most infants with moderate or severe post-asphyctic encephalopathy and which may also influence the neurological outcome.

In the present study our aim was to develop a new model of fetal HI preconditioning by combining two global asphyctic insults which will reflect better the pathological conditions of asphyxia induced in a very early stage of brain development. For this purpose, the uterine vasculature of pregnant rats was clamped for 30 minutes on E17. On P0, the uterine horns still containing the fetuses were placed in a water bath to induce a second perinatal asphyctic insult. On P4 the reactivity of isolated carotid arteries was studied in a wire myograph. On P8, brains were analyzed for apoptotic cell death in the prefrontal cortex and striatum, using a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining. We hypothesize that fetal asphyxia (FA) may alter carotid artery responses and/or the brain damage induced by severe perinatal asphyxia group (SPA) in the rat.

3. MATERIALS AND METHODS

All experiments were approved by Animal Ethics Board of Maastricht University on animal welfare according to Dutch governmental regulations. Sprague-Dawley rats, obtained form Charles River (Maastricht, The Netherlands), were kept under standard laboratory conditions with food and water given ad libitum, 21±2˚C environment temperature, a 12h light/dark schedule (lights on at 07:00 h) and background noise provided by radio. Exclusively male offspring were used within this study, because both morphological and behavioral evidence show a differential vulnerability to a birth insult in males versus females. A greater impact is seen in the male gender, probably due to the protecting role of the circulating estrogens in females (13, 14).

3.1. Animal model

The new preconditioning model is a combination of two previously well described models. The first one is a model for FA, used as a preconditioning stimulus, on E17, by completely clamping the uterine and ovarian arteries with removable clamps for 30 minutes. A second hit, SPA, was induced on E21/22 by submersing the uterine horns still containing the fetuses in a water bath, precisely calibrated at 37ºC, for exactly 19 minutes (starting from cutting off the blood circulation of the uterus to the moment the pups were taken from the water bath) (12, 15). The uterine horns were dissected quickly and the pups were removed, cleaned with medical swipes and stimulated manually to breathe to aid recovery within a pediatric incubator (37ºC, 60-80% humidity, normal gas values). Pups of all conditions were left to recover for approximately 60 minutes after birth in the same controlled environment. No more than 2 pups per litter were used to prevent litter effects. Afterwards, the pups were randomly cross-fostered to surrogate dams, each surrogate mother receiving 10 pups. Four groups of animals were used: FA, SPA, fetal asphyxia combined with severe perinatal asphyxia (FA+SPA) and control. Body weight of the pups was measured on P0 and P8 or P15, respectively.

3.2. Vascular reactivity studies

Common carotid arteries from P4 pups were isolated and kept in Krebs-Ringer bicarbonate (KRB) solution (in mmol/L: NaCl 118.5, MgSO4 * 7 H2O 1.2, KH2PO4 1.2, NaHCO3 25.0, KCl 4.7, CaCl 2.5 and glucose monohydrate 5.5). Rings of +/-2mm in length were mounted between an isometric force transducer (Kistler Morce DSC 6, Seattle, U.S.A.) and a displacement device in a myograph (model 610M, Danish Myotechnology, Aarhus, Denmark) using two stainless steel wires (diameter 40 µm). During mounting and experimentation, the KRB buffer was maintained at 37°C and aerated with 21% O2-74% N2-5% CO2. Artery rings were normalized to a resting pretension corresponding to an intraluminal pressure of 55mmHg. Pilot experiments showed that at this initial passive tension the vessels developed maximal contraction in response to 62.5 mM K+.

Vascular responses to K+ (62,5mM), the thromboxane A2 mimetic U46619 (0.1 microM), the adrenergic receptor agonist norepinephrine (NE, 0.1-100 microM), the endothelium-dependent vasodilator acetylcholine (ACh 0.1-100 microM), the NO-donor sodium nitroprusside (SNP, 0.1-100 microM), the adenylate cyclase stimulator forskolin (0.1-10 microM), and acute hypoxia were studied. For the study of the responsiveness to ACh, SNP, and hypoxia vessels were pre-contracted with U46619 (0.1 microM). Hypoxia was induced by switching the bubbling gas mixture from 21% O2-74% N2-5% CO2 (pO2 17 ± 1 kPa) to 95% N2-5% CO2 (pO2 2.3 ± 0.4 kPa), as previously described (16).

3.3. Evaluation of apoptotic cell death

On P8, pups were anesthetized and perfused intracardially by fixative containing 4% paraformaldehyde. Right hemispheres were entirely cut to serial, 30microm-thick coronal cryostate sections. Sections were dried, defatted with a Triton-X100 solution and stained with cresyl violet. Volumes estimates of the striatum and prefrontal cortex were calculated according to the Cavalieri's principle (17). This principle gives an unbiased estimate of the volume of the region of interest by multiplying the sum of the profile areas on all sections with the distance between the sections. The prefrontal cortex and the striatum were delineated as previously described by Kantor *et al.* (18) by their anatomical landmarks according to Paxinos and Watson (19). Tracing was carried out with a stereology workstation and StereoInvestigator software (MicroBrightField, Williston, VT). Left hemispheres were

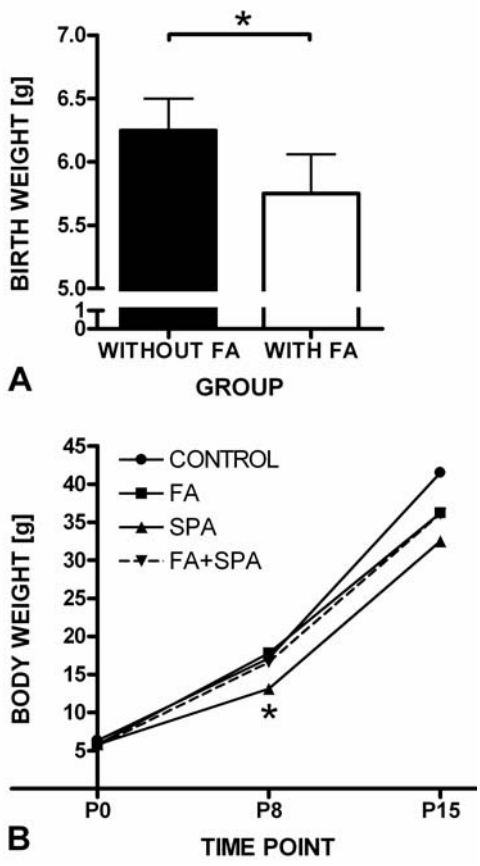

Figure 1. The birth and body weights of the different groups on the different time points**.** (A) Birth weight of the pups. The pups that underwent a FA insult, meaning both the FA and the FA+SPA group, have a smaller birth weight than the pups that did not undergo a FA insult (control and SPA group). (B) Body weights of the pups at the different time points during the experiment. On P8, the mean body weight of the SPA group was lower compared to the other groups. N = 4 animals per group. Data are expressed as mean + SEM. (ANOVA + Bonferroni; * $p<0.05$).

cut into serial, 16microm-thick coronal cryostate sections and cell death was identified by a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining, as described before by Van de Berg *et al* (20). Briefly, sections were permeabilized by methacarn and then labeled with the reaction mixture in a humidified box at 37°C for 1.5h. This reaction mixture consisted of 0.1microl TdT (400U/microl); 1microl cobalt chloride (2.5 mM); 2microl TdT reaction buffer; 0.4microl biotin-dUTP (2 nM); 6.5microl MilliQ (Roche Diagnostics Nederland B.V., Almere, Netherlands). The reaction was stopped by placing the slides in 4x standard saline citrate buffer. Next, streptavidin-Alexa 594 (1:2000 dilution; for 60 min at RT, Sigma, The Netherlands) was used to visualize nuclei labeled with biotinylated dUTP. Sections were counterstained with Hoechst 33342 (1:500 dilution; 30 min at RT; Sigma, The Netherlands). The procedure was finished by washing and enclosure with TBS:glycerol (1:3). TUNEL-positive cells were counted in the two regions of interest (striatum and prefrontal cortex), using the counting method described by Van de Berg *et al.* (20). To detect the presence of positive cells, all slices were examined at a magnification 40x with an Olympus AX-70 microscope. Every TUNEL-positive cell, characterized by its intense red staining, was double-checked with the Hoechst 33342 staining to seen whether that particular cell was highly pyknotic and whether the nucleus was fragmented.

3.4. Statistical analysis

Results are shown as means (SD) of measurements in n animals. For clarity, results in the figures are shown as means +/- SE. Vascular contractions are expressed in terms of active wall tension (N/m), calculated as the force divided by twice the length of the arterial segment, while the relaxant responses are expressed as the percentage of reduction of the contraction induced by U46619. Sensitivity (expressed as pD2= -log EC50) and maximal response (Emax) to agonists was determined for each artery by fitting individual concentration-response data to a non-linear sigmoidal regression curve (Graphpad Prism version 5.01; GraphPad Software Inc, San Diego, U.S.A.). Apoptotic cell death results are expressed in terms of mean total number of TUNEL-positive cells per area as well as density of TUNEL-positive cells per area. Qualitative variables were compared using the Fisher exact test and continuous variables through analysis of variance (ANOVA) followed by a Bonferroni post hoc test. Differences were considered to be significant if $P<0.05$. All calculations were done using the Statistical Package for the Social Sciences (SPSS 15.0 software).

4. RESULTS

4.1. Survival

55% of the SPA animals died at birth, while none of the FA or control animals died. When the pups had a prenatal HI exposure before being exposed to a perinatal insult, fewer animals died (FA+SPA= 36%). This difference in mortality rate between the SPA and the FA+SPA did not reach statistical significance ($p=0.107$; N=243 pups and 25 dams). Moreover, a few pups died in the period between P0 and P15. These differences were, however, not significant. It is also important to note that none of the fetuses died intrauterinely in the period between the FA insult at E17 and birth.

4.2. Somatic growth

The mean birth weight of the pups which underwent FA, i.e. both the FA and FA+SPA groups, was significantly lower than that of the pups that did not undergo the insult (-7.3%; $p=0.044$) (Figure 1A). Body weights were also measured throughout the course of the study, on P8 and P15 (Figure 1B). The decrease in birth weight following FA, seen on P0, had completely disappeared on P8. On that time point, however, the SPA pups showed a significant growth retardation in comparison to the other groups (FA versus FA+SPA; -5.6%; $p=0.048$).

4.3. Reactivity of carotid arteries

Potassium (62,5 mM) and U46619 (0.1 microM) induced tonic contractions in neonatal carotid arteries

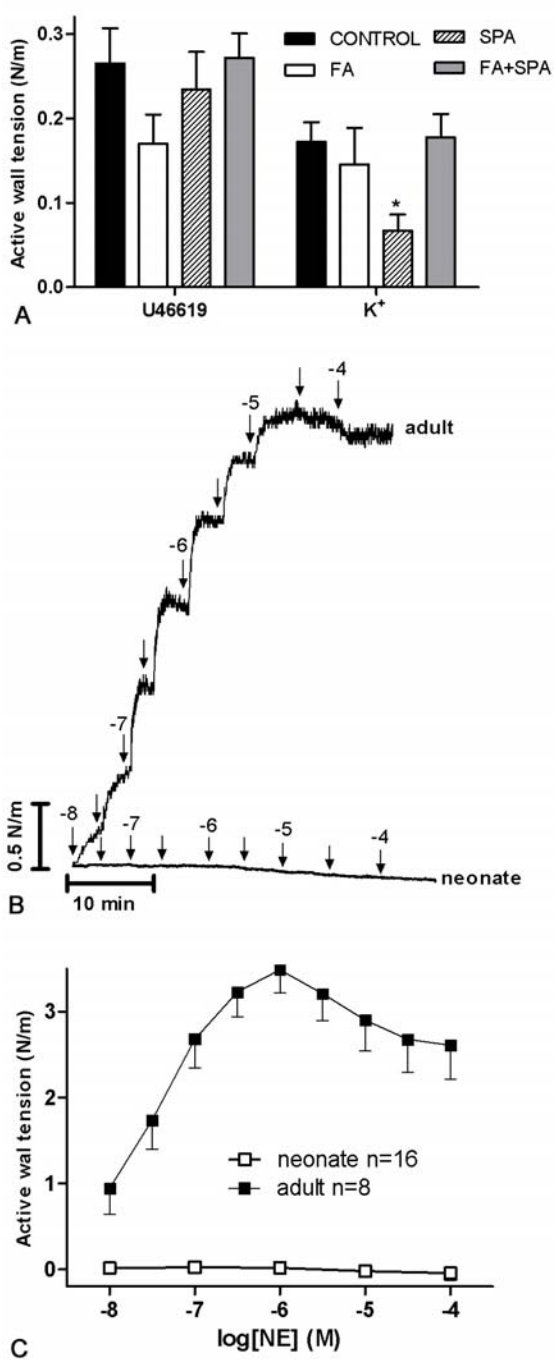

Figure 2. Contractions induced by K+ (62,5mM), the thromboxane A2 mimetic U46619 (0.1 microM), and the adrenergic receptor agonist norepinephrine (NE, 0.1-100 microM) in neonatal (P4) carotid arteries. A: The contraction induced by K+ was significantly reduced in the SPA group (*P<0.05) when compared with the control (CON) and the FA+SPA groups. The contractions induced by U46619 were not significantly different in the four groups. B: Representative tracing showing the contractile effects of NE in neonatal and adult (18-wk-old) carotid arteries. C: Summary of the contractile effects of NE in neonatal and adult carotid arteries.

(Figure 2A). In contrast, NE induced very weak contractions (Control Emax 0.025N/m, SD 0.030, n=16). To asses whether this hyporesponsiveness to NE was an age-related feature, we performed concentration-response curves to NE in carotid arteries from adult rats (18-wk-old) and observed a high contractile potency and efficacy of the adrenergic agonist (Figure 2B and 2C). The contraction induced by K+ was significantly reduced in the SPA group (0.067 N/m, SD 0.072, n=14) when compared with the control (0.182 N/m, SD 0.147, n=37) and the FA+SPA (0.177 N/m, SD 0.117, n=18) groups (Figure 2A). The contractions induced by U46619 were not significantly different in the four groups (Figure 2A).

ACh (Figure 3A), SNP (Figure 3B) and forskolin (Figure 3C) relaxed U46619-contracted neonatal carotid arteries in a concentration-dependent manner. The maximal relaxation induced by ACh was significantly higher in the FA group (Emax 96.79%,SD 70.08, n=5) than in the control (Emax 41.65%, SD 18.99, n=18) and the FA+SPA (Emax 38.53%, SD 24.60, n=10) groups (Figure 3A). Sensitivities to ACh were comparable in the four groups (pD2 control: 6.62, SD 0.46; FA: 6.48, SD 0.15; SPA: 6.49, SD 0.72; FA+SPA: 6.45, SD 0.72). The relaxations induced by SNP and forskolin were not significantly different in the four groups.

Acute hypoxia relaxed U46619-induced contraction (Figure 3D). The maximal relaxations observed after 10 minutes of hypoxia were not significantly different in the four groups (control: 28.99%, SD 22.75; FA: 65.28, SD 79.68; SPA: 16.13, SD 57.31, FA + SPA: 32.30, SD 39.73). Also when the relaxation induced by 10 min of hypoxia was expressed as area above the curve (corrected for the U46619-induced contraction) no significant differences among the four groups were observed (Figure 3E).

4.4. Apoptotic cell death (TUNEL)

No gross morphological changes were seen in the brain of asphyctic pups compared with control pups 8 days after delivery. Typical apoptotic morphological changes like condensation, shrinkage and fragmentation were found in all four groups and were visible with both the TUNEL and the Hoechst staining (Figure 4). TUNEL stained cells were mainly found in the area surrounding the lateral ventricle and the corpus callosum and in the dorsal part of the striatum. They mostly lay together in clusters, showing a patchy pattern.

Figure 5 summarizes the results of the quantitative analysis of the TUNEL stained cells and the volume estimates in the striatum and prefrontal cortex. Two-way ANOVA analysis revealed no significant changes in either striatal or prefrontal cortex volume between groups (Figures 5B and 5E). Two-way ANOVA analysis also revealed a significant increase in the total number of apoptotic cells due to a FA insult (FA and FA+SPA group) in both areas investigated (p=0.03 and p=0.04, respectively). However, post-hoc Bonferroni testing did not detect any significant differences between the FA and FA+SPA groups and the control group (Figures 5A and

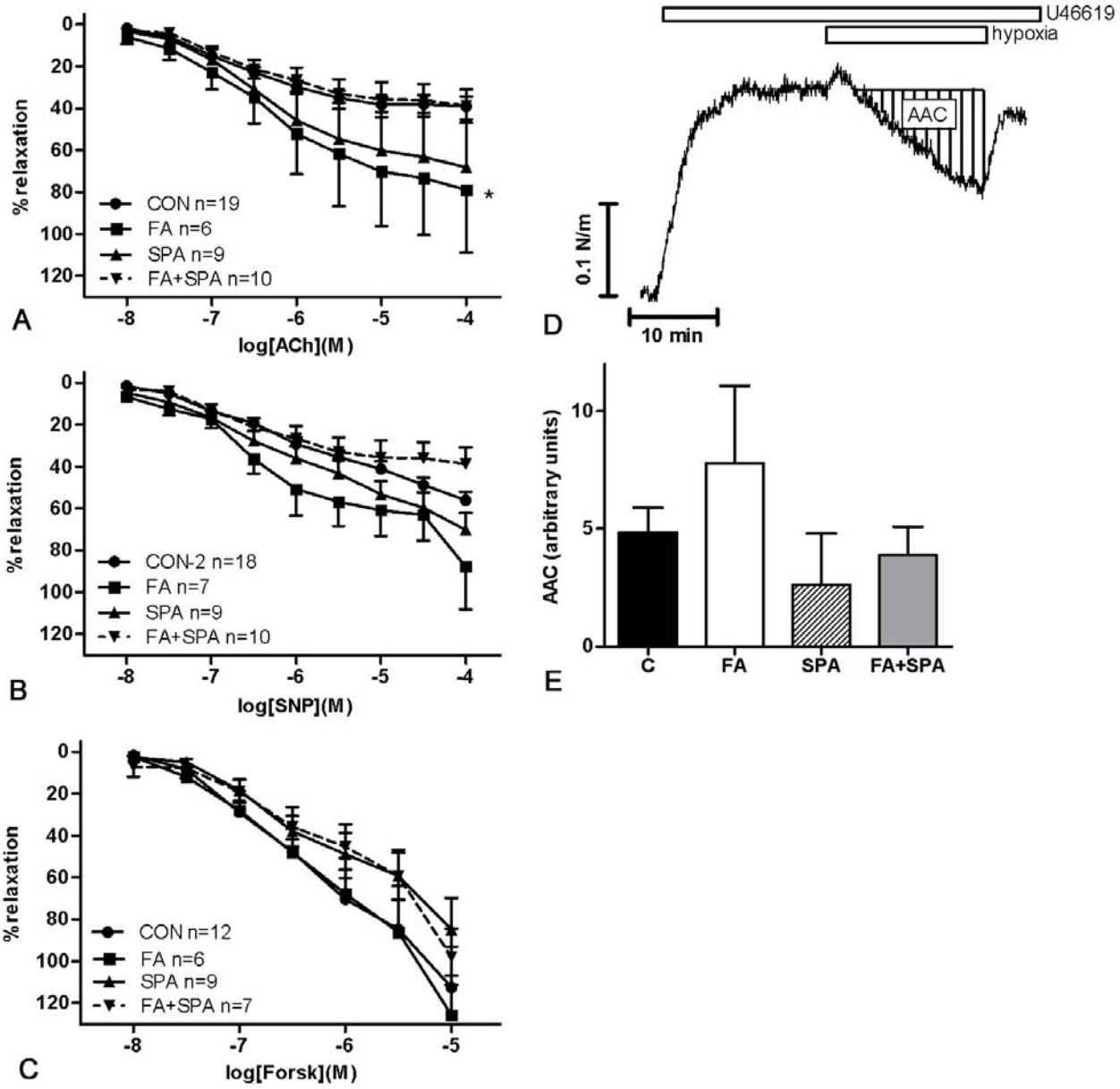

Figure 3. A-C: Concentration-dependent relaxant effects of the endothelium-dependent vasodilator acetylcholine (ACh, A), the NO-donor sodium nitroprusside (SNP B), and the adenylate cyclase stimulator forskolin (C) in neonatal (P4) carotid arteries pre-contracted with U46619 (0.1 microM). The maximal relaxation induced by ACh was significantly higher (*P<0.05) in the FA group than in the control (CON) and the FA+SPA groups. D: Representative tracing showing the effects of a 10 min hypoxic challenge (switching the gas mixture in the organ chamber from 21% O2-74% N2-5% CO2 to 95% N2-5% CO2) in a neonatal carotid artery pre-contracted with U46619 (0.1 microM) . E: Summary of the relaxant responses to acute hypoxia (expressed as area above the curve, AAC) in the four experimental groups.

5D). In both striatum and prefrontal cortex, SPA also significantly increased the total number of apoptotic cells (two-way ANOVA: p=0.01 and p=0.02, respectively). This difference could be almost completely attributed to the SPA (Post-hoc Bonferroni; control vs SPA: p=0.01 and p=0.01, resp.; +127% and +133%, resp.), and not to FA+SPA group. Brains subjected to FA before undergoing SPA demonstrated a significant reduction in apoptotic cells in the striatum (Post-hoc Bonferroni; p<0.001; -46.4%) and prefrontal cortex (Post-hoc Bonferroni; p=0.003; -44%) compared to brains only subjected to SPA.

Taking into account both the data of the TUNEL-positive cell counting and the volume measurements allowed us to estimate the density of apoptotic cells (Figures 5C and 5F). FA caused a small increase in the

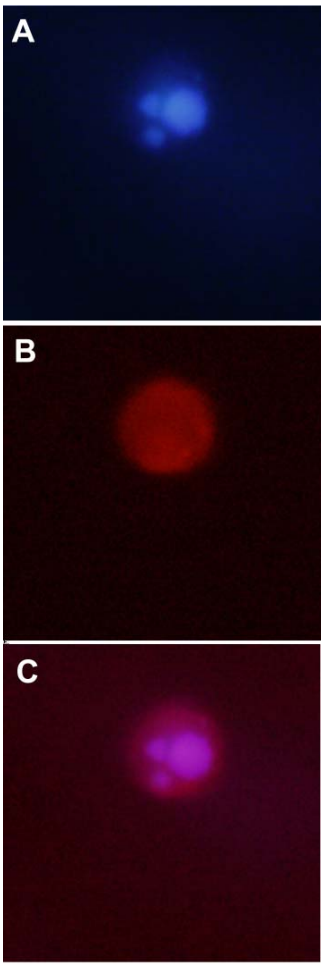

Figure 4. Representing photographs of a TUNEL-stained cell. (A) Hoechst stained section at a high magnification (100x) (B) TUNEL stained section. (C) Hoechst and TUNEL dubbel stained section.

mean density of apoptotic cells in the striatum and the prefrontal cortex (Two-way ANOVA: $p=0.02$ and $p=0.04$, resp.). However, post-hoc Bonferroni analysis did not find any significant differences between the FA group and the control group. Two-way ANOVA also revealed a significant increase in the mean density of apoptotic cells due to SPA in both areas ($p<0.001$ and $p=0.003$, resp.), which could be almost entirely ascribed to the SPA group (Post-hoc Bonferroni; control vs SPA: $p<0.001$ and $p<0.001$, resp.; +138% and +166%). Moreover, post-hoc testing demonstrated that the mean density of apoptotic cells in the FA+SPA group was significantly lower than in the SPA group in the striatum ($p<0.001$; -33%) as well as the prefrontal cortex ($p=0.007$; -47%).

5. DISCUSSION

Asphyxia during development is a major cause of neonatal mortality and of lifelong neurological disabilities among survivors. Although recent evidence suggests that moderate hypothermia (core temperature 33-34°C for 72 hours) improves the outcome of term newborns that suffered from moderate post-asphyctic encephalopathy (21), there are still no other clinically proven neuroprotective agents available for these newborns. Revealing the mechanisms behind HI preconditioning and tolerance could therefore be an important step towards an effective therapy. We aimed to determine whether a fetal asphyctic insult protects against a subsequent more severe perinatal asphyctic insult. Both insults used are global events that are associated with both systemic metabolic and hemodynamic changes in the fetus that may influence the outcome of the animal. These changes mimic the global and pathophysiological conditions of asphyxia induced before and during birth. Therefore, they reflect the clinical situation more closely than stroke insults. In the present study, we evaluated the effects of this novel model of preconditioning on carotid vascular reactivity and cerebral apoptotic cell death.

5.1. Vascular reactivity studies

Following severe perinatal asphyxia the autoregulatory ability of the neonatal cerebral vascular bed is impaired (22, 23) . We aimed to analyze whether this impairment was reflected in alterations of neonatal carotid arteries reactivity. The carotid arteries play an important role in responses to hypoxia, particularly in the fetal and neonatal period, where the regulatory capacity of the small cerebral arteries is functionally immature (24). We have observed that neonatal (P4) rat carotid arteries are sensitive to receptor-independent (high K+) and –dependent (U46619) contractile agonists as well as to endothelium-dependent (ACh) and –independent (SNP and forskolin) relaxant agonists. Moreover, carotid responsiveness to acute hypoxia was also present at this age. Interestingly, the P4 carotid arteries did not respond to the adrenergic agonist NE, which was a potent contractile agent in the adult carotid arteries.

The switch from the proliferative to the mature contractile vascular phenotype is an important developmental event whose timing appears to be vessel and species specific (25-27) . This process include maturational changes in contractile proteins, ion channels, endothelial cell function, vessel wall growth and receptor expression. In rodents an important part of these maturational changes appears to occur postnatally (25), whereas it begins before birth in sheep (26). Accordingly, several studies demonstrated adrenergic-mediated contractions in isolated carotid arteries from fetal and neonatal lambs (26, 28) (29). In fetal and neonatal rat carotid arteries, mounted in a pressurized myograph system, adrenergic agonists induced some reduction in lumen diameter (30, 31). However our present data indicate that these diameter changes are probably not accompanied by a significant increase in carotid wall tension. In contrast, the vascular contractile machinery was mature to respond to non-adrenergic agonists. Fetal and perinatal asphyxia are stressors that elicit integrated cardiovascular and endocrine responses producing a redistribution of the combined ventricular output in favor of the adrenal, myocardial, and cerebral circulations (32). Rat and lamb models are the most frequently used in the study of the pathophysiology of

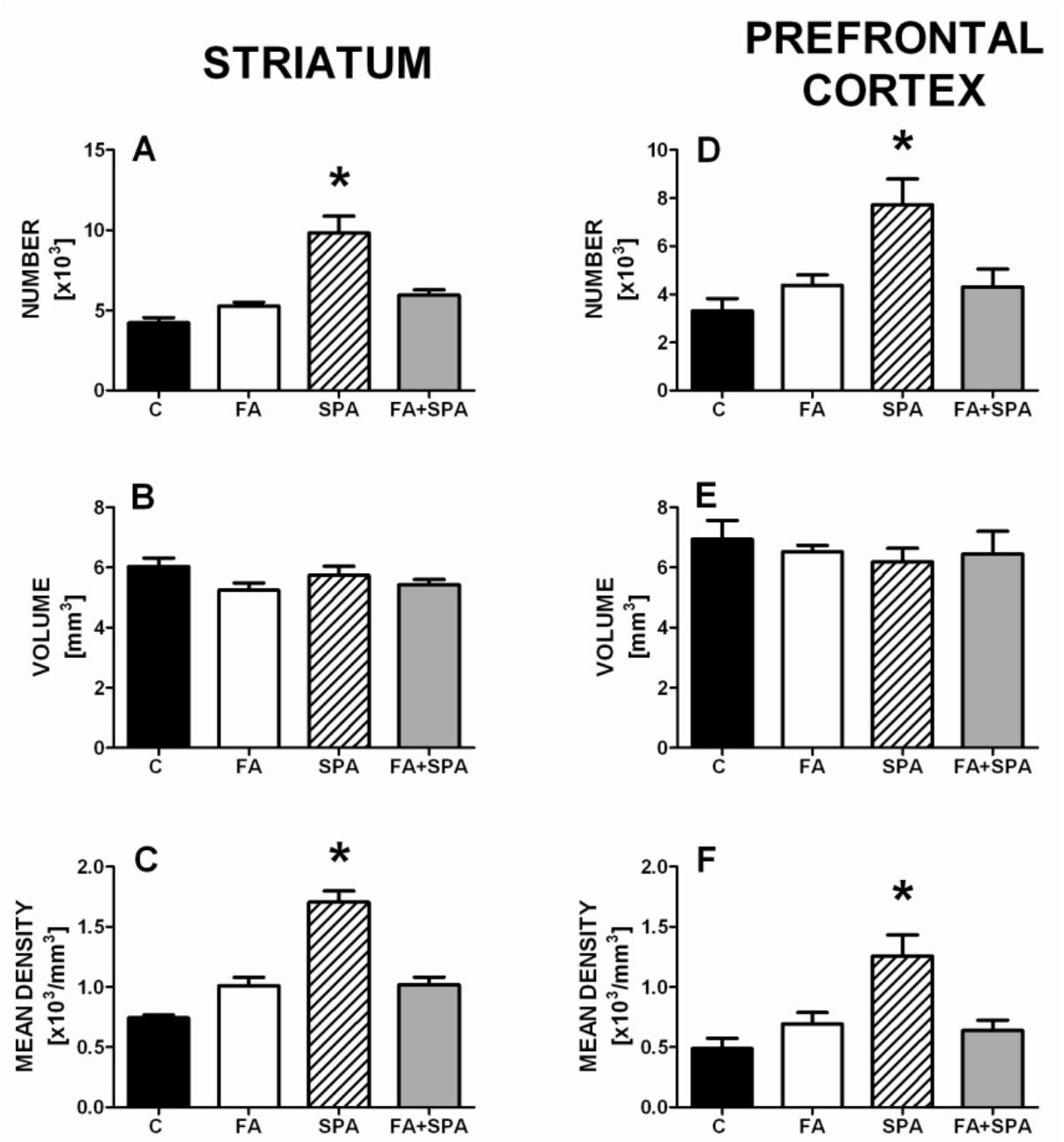

Figure 5. Quantitative analysis of TUNEL-positive cells and volume measurements of the striatum (A-C) and the prefrontal cortex (D-F) 8 days after the severe perinatal insult. (A) The total number of apoptotic cells in the striatum (x103). There is a significant increase in the number of TUNEL-positive cells in the striatum of SPA animals compared to the other groups. In addition, FA significantly decreased the number of apoptotic cells after SPA. (B) Striatal volume estimates (mm3). There was no significant difference in striatal volume between groups. (C) The mean density of apoptotic cells in the striatum (x103/mm3). The mean density of TUNEL-positive cells was higher in the SPA group than in the other groups. Furthermore, this density was significantly lower in the FA+SPA group compared to SPA group. (D) The total number of apoptotic cells in the prefrontal cortex (x103). SPA group had a significantly more TUNEL-positive cells than the other groups. FA+SPA pups had less TUNEL-positive cells than pups only subjected to SPA. (E) Volume estimates of the prefrontal cortex (mm3). The prefrontal cortex volume estimates did not differ between groups. (F) The mean density of apoptotic cells in the prefrontal cortex (x103/mm3). The SPA group significantly higher the mean density of TUNEL-positive cells compared to the other three groups. Additionally, FA lowered the mean density of TUNEL-positive cells after SPA. N = 4 animals per group. Data are expressed as mean + SEM. (ANOVA + Bonferroni; * $p<0.05$).

fetal and perinatal asphyxia. The differential maturation in vascular responsiveness between the two species, particularly in adrenergic responsiveness, should be taken into account when interpreting the flow redistribution response induced by asphyxia.

When we analyzed the effects of fetal and perinatal asphyxia on carotid reactivity, we observed a reduced receptor-independent contraction in the SPA group and an increased endothelium-dependent relaxation in the FA group. A similar trend, although not significant, toward increased endothelium-dependent relaxation was observed in the SPA group. Interestingly, when the animals were exposed to fetal and perinatal asphyxia, carotid artery responsiveness was similar to the observed in the control group. These findings indicate that functional changes occur in neonatal vascular reactivity following asphyxia, a result that may significantly influence asphyctic damage by altering blood flow to the brain. Endothelium-derived nitric oxide (NO) plays an important role in the autoregulation of cerebral circulation and numerous experimental evidences indicate its involvement in post HI brain injury (22, 33-38). Excessive NO production leads to damage to the cerebral microcirculation and directly to brain tissue (33, 38, 39). In addition, immediate postischemic cerebral hyperperfusion appears to be mediated, at least partially, by increased NO production and postischemic reperfusion damage to the brain can be reduced by NO synthase blockade (22, 34-37). Our present data suggest that exposure to fetal asphyxia-induced preconditioning might modulate the NO regulatory ability on post HI cerebral blood flow.

5.2. Apoptotic cell death

To evaluate the putative brain protective effect of fetal asphyctic preconditioning, the number of apoptotic cells was analyzed in the striatum and prefrontal cortex of 8-day-old rat pups, using a TUNEL staining. The TUNEL method detects nuclear DNA fragmentation in cells. Although fragmentation is a pattern typical of apoptosis, it can also occur, to a lesser extent, in necrotic cells. As a consequence, TUNEL can sometime also stain necrotic cells. Because each stained cell was analyzed for apoptotic traits like nuclear fragmentation and a pyknotic nucleus with a Hoechst staining, we assume that the majority of counted cells were apoptotic.

Only a few apoptotic cells were found in the control and FA group. This kind of apoptosis probably represents naturally occurring developmental cell death. The SPA group showed a substantial increase in TUNEL-positive cells in both the striatum and prefrontal cortex. Pups that underwent FA before being subjected to SPA had significantly less apoptotic cells than pups that did not undergo FA, suggesting a protective effect. Their number of apoptotic cells was similar to that of the control and FA groups. Since the results were similar for both areas investigated (i.e. prefrontal cortex and striatum), it seems that both the damage and the protection represent a global effect. Another important finding of this study is that the SPA group showed a growth retardation which was absent in the FA+SPA group. Since the FA insult was global, this protection may include protective effects of many organs and systems, such as the heart and the arteriovenous system. This awaits further research though.

Although this study is the first to look at HI preconditioning in a global asphyxia model, our current findings are consistent with cell protection by HI preconditioning against focal HI injury (i.e. stroke models). Cantagrel *et al.* found that exposure of the pregnant dam to a hypoxic gas mixture (8%O2/92%N2) causes a reduction in the number of apoptotic cells in the striatum, hippocampus and cortex 24h and 48h after right carotid ligation on postnatal day 7 (Rice-Vannucci model) (40). In the same rat model, Zhao *et al.* demonstrated that newborns subjected to a sublethal prenatal asphyxia show lower mortality rates, less brain tissue damage, less neuronal loss and fewer caspase-3 positive cells after stroke (41). Similarly, Cai *et al.* demonstrated that a prenatal HI insult, achieved by clamping the ovarian and uterine arteries for 30 min, also reduces the brain infarct size and neuronal injury induced by the Rice-Vannucci model (12).

An important remark to be made is that the neuroprotective effects induced by fetal asphyxia in this study may be partly due to the anesthetics (i.e. isoflurane). Kersten *et al.* reported that isoflurane can mimic the protecting effects of preconditioning in the rat myocardium, while Kapinya *et al* showed that isoflurane induces prolonged protection against cerebral ischemia (42, 43). Moreover, Caesarean section per se induces specific neurobehavioral alterations with respect to, for example, response to stress. Furthermore, the addition of a period of asphyxia worsens some of the behavioral changes produced by C-sections alone. Consequently, isoflurane and C-section can possibly influence the results of apoptotic cell counts. For that reason, it is important to include vaginally-delivered as well as SHAM-operated control animals in further studies.

5.3. Final remarks

Animals exposed to a brief period of global FA seem to be protected against the vascular alterations and the increased apoptotic cell death in striatum and prefrontal cortex, following SPA 4 days later. This FA preconditioning is most likely the result of the delayed phase of neuroprotection, which requires new protein synthesis and gene expression. This anti-apoptotic effect probably involves several molecules, as described above, that are induced and regulated as part of a complex. To imply the role of these molecules in FA-induced tolerance, further experiments are required. Identifying these mechanisms could provide rational targets for future pharmacological intervention.

6. REFERENCES

1. Low, J. A.: Determining the contribution of asphyxia to brain damage in the neonate. *J Obstet Gynaecol Res*, 30, 276-86 (2004)

2. Low, J. A.: Reflections on the occurrence and significance of antepartum fetal asphyxia. *Best Pract Res Clin Obstet Gynaecol*, 18, 375-82 (2004)

3. Marlow, N., A. S. Rose, C. E. Rands & E. S. Draper: Neuropsychological and educational problems at school age associated with neonatal encephalopathy. *Arch Dis Child Fetal Neonatal Ed*, 90, F380-7 (2005)

4. Lawn, J. E., S. Cousens, Z. A. Bhutta, G. L. Darmstadt, J. Martines, V. Paul, R. Knippenberg, H. Fogstadt, P. Shetty & R. Horton: Why are 4 million newborn babies dying each year? *Lancet*, 364, 399-401 (2004)

5. Rennie, J. M., C. F. Hagmann & N. J. Robertson: Outcome after intrapartum hypoxic ischaemia at term. *Semin Fetal Neonatal Med*, 12, 398-407 (2007)

6. Barrett, R. D., L. Bennet, J. Davidson, J. M. Dean, S. George, B. S. Emerald & A. J. Gunn: Destruction and reconstruction: hypoxia and the developing brain. *Birth Defects Res C Embryo Today*, 81, 163-76 (2007)

7. Tanaka, H., H. Yokota, T. Jover, I. Cappuccio, A. Calderone, M. Simionescu, M. V. Bennett & R. S. Zukin: Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. *J Neurosci*, 24, 2750-9 (2004)

8. Kitagawa, K., M. Matsumoto, M. Tagaya, R. Hata, H. Ueda, M. Niinobe, N. Handa, R. Fukunaga, K. Kimura, K. Mikoshiba & *et al.*: 'Ischemic tolerance' phenomenon found in the brain. *Brain Res*, 528, 21-4 (1990)

9. Stagliano, N. E., M. A. Perez-Pinzon, M. A. Moskowitz & P. L. Huang: Focal ischemic preconditioning induces rapid tolerance to middle cerebral artery occlusion in mice. *J Cereb Blood Flow Metab*, 19, 757-61 (1999)

10. Plaschke, K., M. A. Weigand, A. Michel, E. Martin & H. J. Bardenheuer: Permanent cerebral hypoperfusion: 'preconditioning-like' effects on rat energy metabolism towards acute systemic hypotension. *Brain Res*, 858, 363-70 (2000)

11. Gidday, J. M., J. C. Fitzgibbons, A. R. Shah & T. S. Park: Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. *Neurosci Lett*, 168, 221-4 (1994)

12. Cai, Z., J. D. Fratkin & P. G. Rhodes: Prenatal ischemia reduces neuronal injury caused by neonatal hypoxia-ischemia in rats. *Neuroreport*, 8, 1393-8 (1997)

13. El-Khodor, B. F. & P. Boksa: Differential vulnerability of male versus female rats to long-term effects of birth insult on brain catecholamine levels. *Exp Neurol*, 182, 208-19 (2003)

14. Zhang, Y. Q., J. Shi, G. Rajakumar, A. L. Day & J. W. Simpkins: Effects of gender and estradiol treatment on focal brain ischemia. *Brain Res*, 784, 321-4 (1998)

15. Van de Berg, W. D., A. Blokland, A. C. Cuello, C. Schmitz, W. Vreuls, H. W. Steinbusch & C. E. Blanco: Perinatal asphyxia results in changes in presynaptic bouton number in striatum and cerebral cortex-a stereological and behavioral analysis. *J Chem Neuroanat*, 20, 71-82 (2000)

16. Cogolludo, A., L. Moreno, G. Frazziano, J. Moral-Sanz, C. Menendez, J. Castaneda, C. Gonzalez, E. Villamor & F. Perez-Vizcaino: Activation of neutral sphingomyelinase is involved in acute hypoxic pulmonary vasoconstriction. *Cardiovasc Res* (2008)

17. Schmitz, C. & P. R. Hof: Design-based stereology in neuroscience. *Neuroscience*, 130, 813-31 (2005)

18. Kantor, O., Y. Temel, C. Holzmann, K. Raber, H. P. Nguyen, C. Cao, H. O. Turkoglu, B. P. Rutten, V. Visser-Vandewalle, H. W. Steinbusch, A. Blokland, H. Korr, O. Riess, S. von Horsten & C. Schmitz: Selective striatal neuron loss and alterations in behavior correlate with impaired striatal function in Huntington's disease transgenic rats. *Neurobiol Dis*, 22, 538-47 (2006)

19. Paxinos, G. & C. Watson: The rat brain in stereotaxic coordinates. Academic press, San Diego (1982)

20. Van de Berg, W. D., C. Schmitz, H. W. Steinbusch & C. E. Blanco: Perinatal asphyxia induced neuronal loss by apoptosis in the neonatal rat striatum: a combined TUNEL and stereological study. *Exp Neurol*, 174, 29-36 (2002)

21. Hoehn, T., G. Hansmann, C. Buhrer, G. Simbruner, A. J. Gunn, J. Yager, M. Levene, S. E. Hamrick, S. Shankaran & M. Thoresen: Therapeutic hypothermia in neonates. Review of current clinical data, ILCOR recommendations and suggestions for implementation in neonatal intensive care units. *Resuscitation*, 78, 7-12 (2008)

22. Dorrepaal, C. A., P. Steendijk, J. Baan & F. van Bel: Inhibition of nitric oxide synthesis following severe hypoxia-ischemia restores autoregulation of cerebral blood flow in newborn lambs. *Early Hum Dev*, 60, 159-170 (2001)

23. Lou, H. C., N. A. Lassen & B. Friis-Hansen: Impaired autoregulation of cerebral blood flow in the distressed newborn infant. *J Pediatr*, 94, 118-21 (1979)

24. Williams, J. M., C. R. White, M. M. Chang, E. R. Injeti, L. Zhang & W. J. Pearce: Chronic hypoxic decreases in soluble guanylate cyclase protein and enzyme activity are age dependent in fetal and adult ovine carotid arteries. *J Appl Physiol*, 100, 1857-66 (2006)

25. Yoshida, T. & G. K. Owens: Molecular determinants of vascular smooth muscle cell diversity. *Circ Res*, 96, 280-91 (2005)

26. Hutanu, C., B. E. Cox, K. DeSpain, X. T. Liu & C. R. Rosenfeld: Vascular development in early ovine gestation: carotid smooth muscle function, phenotype, and biochemical markers. *Am J Physiol Regul Integr Comp Physiol*, 293, R323-33 (2007)

27. Rzucidlo, E. M., K. A. Martin & R. J. Powell: Regulation of vascular smooth muscle cell differentiation. *J Vasc Surg*, 45 Suppl A, A25-32 (2007)

28. Su, C., J. A. Bevan, N. S. Assali & C. R. Brinkman, 3rd: Development of neuroeffector mechanisms in the carotid artery of the fetal lamb. *Blood Vessels*, 14, 12-24 (1977)

29. Longo, L. D., N. Ueno, Y. Zhao, W. J. Pearce & L. Zhang: Developmental changes in alpha 1-adrenergic receptors, IP3 responses, and NE-induced contraction in cerebral arteries. *Am J Physiol*, 271, H2313-9 (1996)

30. Campbell, M. E., S. J. Williams, S. Veerareddy & S. T. Davidge: Maternal nutrient restriction reduces carotid artery constriction without increasing nitric oxide synthesis in the late gestation rat fetus. *Pediatr Res*, 58, 840-4 (2005)

31. Williams, S. J., M. E. Campbell, I. C. McMillen & S. T. Davidge: Differential effects of maternal hypoxia or nutrient restriction on carotid and femoral vascular function in neonatal rats. *Am J Physiol Regul Integr Comp Physiol*, 288, R360-7 (2005)

32. Fletcher, A. J., D. S. Gardner, C. M. Edwards, A. L. Fowden & D. A. Giussani: Development of the ovine fetal cardiovascular defense to hypoxemia towards full term. *Am J Physiol Heart Circ Physiol*, 291, H3023-34 (2006)

33. Beckman, J. S.: The double-edged role of nitric oxide in brain function and superoxide-mediated injury. *J Dev Physiol*, 15, 53-9 (1991)

34. Clavier, N., J. R. Kirsch, P. D. Hurn & R. J. Traystman: Effect of postischemic hypoperfusion on vasodilatory mechanisms in cats. *Am J Physiol*, 267, H2012-8 (1994)

35. Clavier, N., J. R. Kirsch, P. D. Hurn & R. J. Traystman: Cerebral blood flow is reduced by N omega-nitro-L-arginine methyl ester during delayed hypoperfusion in cats. *Am J Physiol*, 267, H174-81 (1994)

36. Dorrepaal, C. A., M. Shadid, P. Steendijk, E. T. Van der Velde, M. Van de Bor, J. Baan & F. Van Bel: Effect of post-hypoxic-ischemic inhibition of nitric oxide synthesis on cerebral blood flow, metabolism and electrocortical brain activity in newborn lambs. *Biol Neonate*, 72, 216-26 (1997)

37. Greenberg, R. S., M. A. Helfaer, J. R. Kirsch & R. J. Traystman: Effect of nitric oxide synthase inhibition on postischemic cerebral hyperemia. *Am J Physiol*, 269, H341-7 (1995)

38. Sato, S., T. Tominaga, T. Ohnishi & S. T. Ohnishi: Electron paramagnetic resonance study on nitric oxide production during brain focal ischemia and reperfusion in the rat. *Brain Res*, 647, 91-6 (1994)

39. Perlman, J. M.: Intervention strategies for neonatal hypoxic-ischemic cerebral injury. *Clin Ther*, 28, 1353-65 (2006)

40. Cantagrel, S., C. Krier, S. Ducrocq, S. Bodard, V. Payen, J. Laugier, D. Guilloteau & S. Chalon: Hypoxic preconditioning reduces apoptosis in a rat model of immature brain hypoxia-ischaemia. *Neurosci Lett*, 347, 106-10 (2003)

41. Zhao, P. & Z. Zuo: Prenatal hypoxia-induced adaptation and neuroprotection that is inducible nitric oxide synthase-dependent. *Neurobiol Dis*, 20, 871-80 (2005)

42. Kapinya, K. J., K. Prass & U. Dirnagl: Isoflurane induced prolonged protection against cerebral ischemia in mice: a redox sensitive mechanism? *Neuroreport*, 13, 1431-5 (2002)

43. Kersten, J. R., T. J. Schmeling, P. S. Pagel, G. J. Gross & D. C. Warltier: Isoflurane mimics ischemic preconditioning via activation of K (ATP) channels: reduction of myocardial infarct size with an acute memory phase. *Anesthesiology*, 87, 361-70 (1997)

Abbreviations: E: embryonic day, FA: fetal asphyxia, P: postnatal day, SPA: severe perinatal asphyxia

Key Words: Preconditioning, Fetal asphyxia, Perinatal asphyxia, Apoptosis, Carotid reactivity

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