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Impaired Cerebral Blood Flow Autoregulation During Postraumatic Arterial Hypotension After Fluid Percussion Brain Injury is Prevented by Phenylephrine in Female but Exacerbated in Male Piglets by ERK MAPK Upregulation

William M. Armstead, Ph.D[1,2], **J. Willis Kiessling, B.A.**[1], **W. Andrew Kofke, M.D.**[1], and **Monica S. Vavilala, M.D.**[3]

[1]Department of Anesthesiology and Critical Care, University of Pennsylvania, Philadelphia, PA l9l04

[2]Department of Pharmacology, University of Pennsylvania, Philadelphia, PA l9l04

[3]Department of Anesthesiology, Pediatrics, and Neurological Surgery, University of Washington, Seattle, WA

Abstract

Objective—Traumatic brain injury (TBI) contributes to morbidity and mortality in children and boys are disproportionately represented. Hypotension is common and worsens outcome after TBI. Extracellular signal-related kinase (ERK) mitogen activated protein kinase (MAPK) is upregulated and reduces CBF after fluid percussion brain injury (FPI) in piglets. We hypothesized that increased CPP via phenylephrine (PHE) sex dependently reduces impairment of cerebral autoregulation during hypotension after FPI through modulation of ERK MAPK.

Design—Prospective, randomized animal study.

Setting—University laboratory.

Subjects—Newborn (1–5 day old) pigs.

Interventions—CBF, pial artery diameter, ICP and autoregulatory index (ARI) were determined before and after FPI in untreated, pre- and post-injury PHE (1 μg/kg/min iv) treated male and female pigs during normotension and hemorrhagic hypotension. CSF ERK MAPK was determined by ELISA.

Measurements and Main Results—Reductions in pial artery diameter, CBF, CPP and elevated ICP after FPI were greater in males, which were blunted by PHE pre- or post-FPI. During hypotension and FPI, pial artery dilation was impaired more in males. PHE decreased impairment of hypotensive pial artery dilation after FPI in females, but paradoxically caused vasoconstriction after FPI in males. Papaverine induced pial artery vasodilation was unchanged by FPI and PHE. CBF, CPP, and ARI decreased markedly during hypotension and FPI in males but less in females. PHE prevented reductions in CBF, CPP, and ARI during hypotension in females but increased reductions in males. CSF ERK MAPK was increased more in males than females after FPI. PHE blunted ERK MAPK upregulation in females, but increased ERK MAPK upregulation in males after FPI.

Reprints will be ordered Address for reprints: William M. Armstead, Ph.D, Department of Anesthesiology and Critical Care, 3620 Hamilton Walk, JM3, University of Pennsylvania, Philadelphia, PA l9l04, (215) 573-3674, Fax: (215) 349-5078, armsteaw@uphs.upenn.edu.

Conclusions—These data indicate that elevation of CPP with PHE sex dependently prevents impairment of cerebral autoregulation during hypotension after FPI through modulation of ERK MAPK. These data suggest the potential role for sex dependent mechanisms in cerebral autoregulation after pediatric TBI.

Keywords

cerebral circulation; pediatric; traumatic brain injury; signal transduction; autoregulation

Introduction

Pediatric traumatic brain injury (pTBI) is a global public health concern (1). Boys are disproportionately represented and young children have devastating outcomes (1). Systemic hypotension is common after TBI (2), results in low cerebral perfusion pressure (CPP) and is thought to cause cerebral ischemia and poor outcome, particularly when cerebral autoregulation is impaired (3,4). Since cerebral blood flow (CBF) is dependent on CPP with impaired cerebral autoregulation and CBF may contribute to neuronal cell integrity, optimal CPP management limits tissue hypoxia from low CPP. Elevation of systemic mean arterial blood pressure (MAP) with a commonly used vasopressor such as intravenous phenylephrine may decrease brain edema (5), increase CPP and CBF, and improve cerebral autoregulation, thereby improving outcome. Since ethical constraints preclude mechanistic studies of cerebral autoregulation in children, we used an established porcine model of fluid percussion injury (FPI) that mimics many of the pathophysiological features of pTBI to corroborate clinical observations after pTBI (6). Piglets offer the unique advantage of a gyrencepahalic brain containing substantial white matter, which is more sensitive to ischemic/TBI damage, similar to humans. Our data suggest that the newborn pig is more cerebrohemodynamically sensitive to FPI compared to the juvenile and impaired autoregulation post insult may be caused by an age dependent decreased production of calcitonin gene related peptides (CGRP) (6–8). Exogenous administration of adrenomedullin, a member of the CGRP family, prevents impairment of cerebral autoregulation after FPI in piglets (8).

Adult data suggest a neuroprotective role of ovarian hormones in females after stroke and TBI (9–11), but little is known in pTBI. Of the 3 published studies (12–14), the last 2 report male gender to be a risk factor for reduced speed and efficiency of information processing after pTBI. Male neonatal cortical astrocytes are more sensitive to oxygen-glucose deprivation than female cells (15), suggesting sex differences in sensitivity to ischemia (16). We have reported more blunted pial artery dilation during hypotension in male vs. female pigs after FPI (8,17).

Mitogen activated protein kinase (MAPK) is a family of at least 3 kinases (extracellular signal-related kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) (18). After FPI, ERK MAPK is upregulated and contributes to reductions in CBF in the newborn pig (19). Therefore, we tested the hypothesis that increased CPP via phenylephrine infusion sex dependently reduces impairment of cerebral autoregulation during hemorrhagic hypotension after FPI through modulation of ERK MAPK in piglets.

Materials and Methods

Closed cranial window and brain injury procedures

Newborn pigs (1–5 days old, 1.0–1.4 Kg) of both sexes were studied. This age of pig roughly approximates that of the six month old – 1 year old human child (20). All protocols were approved by the Institutional Animal Care and Use Committee of the University of

Pennsylvania. Animals were anesthetized with isoflurane (1–2 MAC) and maintained with a-chloralose (30–50 mg/kg. supplemented with 5 mg /kg/h i.v.). A catheter was inserted into a femoral artery to monitor blood pressure and to sample blood gas tensions and pH. The trachea was cannulated, and the animals were ventilated with room air. A heating pad was used to maintain the animals at 37° – 39° C, monitored rectally.

A cranial window was placed in the parietal skull of these anesthetized animals. The closed crainal window technique for measuring pial artery diameter and collection of CSF for ELISA analysis has been previously described (8,17). An Integra Camino monitor was used to measure ICP, the probe being placed in the parietal cortex ipsilateral to the injury site. CBF was measured in the cerebral cortex using radioactively labeled microspheres (17). Briefly, a known amount of radioactivity in 15-μm microspheres (300,000–800,000 spheres) was injected into the left ventricle. After each experiment, the pig was sacrificed and the brain removed and weighed. CBF was determined by counting cerebral cortex brain tissue samples in a gamma counter. The energy from each nuclide was separated by differential spectroscopy. Aliquots of the actual microsphere solutions injected were used for overlap calculations. The count in each milliliter per minute of blood flow was determined by dividing the counts in the reference withdrawal by the rate of reference withdrawal. Thus blood flow can be calculated as $Q = C \times R \times CR^{-1}$, where Q is brain blood flow (in ml/min), C is counts per minute (cpm) in the tissue sample, R is the rate of withdrawal of reference blood sample (in ml/min), and CR is the total counts in the reference blood sample. CBF so determined reflect flow to the cerebral cortex both ipsilateral and contralateral to the injury site.

The method used to induce brain FPI has been described previously (17). A device designed by the Medical College of Virginia was used. A small opening was made in the parietal skull contralateral to the cranial window. A metal shaft was sealed into the opening on top of intact dura and fluid coupled to the brain injury device. The intensity of the injury was 1.9–2.2 atm. with a constant duration of 19–23 ms.

Protocol

Two types of pial vessels, small arteries (resting diameter, 120–160 μm) and arterioles (resting diameter, 50–70 μm) were examined to determine whether segmental differences in the effects of FPI could be identified. Typically, 2–3 ml of artificial CSF were flushed through the window over a 30s period, and excess CSF was allowed to run off through one of the needle ports. For sample collection, 300 μl of the total cranial window volume of 500 μl was collected by slowly infusing artificial CSF into one side of the window and allowing the CSF to drip freely into a collection tube on the opposite side.

Twelve experimental groups were studied (all n=5) (1 and 2) male and female sham control, (3 and 4) male and female FPI, vehicle pre-treated, (5 and 6) male and female FPI pre-treated with phenylephrine (PHE) (1 μg/Kg/min iv), (7 and 8) male and female FPI pre-treated with the ERK MAPK antagonist U 0126 (1 mg/kg iv), (9 and 10) male and female FPI, vehicle post-treated, and (11 and 12) male and female FPI post-treated with PHE. The dose of phenylephrine was chosen based on that used clinically for treatment of pediatric TBI patients at the University of Washington (unpublished observations). In all groups hypotension was induced by the rapid withdrawal of either 5–8 or 10–15 ml blood/Kg to induce moderate or severe hypotension (decreases in mean arterial blood pressure of 25 and 45%, respectively) (21). Such decreases in blood pressure were maintained constant for 10 min by titration of additional blood withdrawal or blood reinfusion (21). The vehicle for all agents was 0.9% saline, except for the MAPK inhibitor, which used dimethyl sulfoxide (100 μl) diluted with 9.9 ml 0.9% saline. In sham control animals, responses to hypotension (moderate, severe) and papaverine (10^{-8}, 10^{-6} M) were obtained initially and then again 1

and 4h later in the presence of the agent vehicle. In drug pre-treated FPI animals, PHE or U 0126 were administered 30 min before FPI and the responses to hypotension and papaverine obtained at 1 and 4h after injury. In drug post-treated animals, PHE was administered 30 min after FPI. The order of agonist administration was randomized within animal drug treatment groups.

Determination of Autoregulatory Index (ARI) via transcranial Doppler (TCD) ultrosonography

A Spencer Technologies PMF-100 TCD monitor was used to insonate the cerebral vessels prior to and after FPI in the absence and presence of induced hypotension and to measure the middle and posterior cerebral artery blood velocity (V_{mca} and V_{pca}) and calculate the autoregulatory index (ARI). By definition, ARI = %Δ CVR / %Δ CPP where CVR ≈ $V_{cerebral\ blood\ vessel}$ / CPP (mean arterial blood pressure, MAP-ICP). ARI < 0.4 = impaired cerebral autoregulation, ARI > 0.4 infers intact cerebral autoregulation, ARI 0 = absent autoregulation and ARI 1.0 reflects perfect autoregulation. An Integra Camino monitor was used to measure ICP and ARI was calculated using CPP in all animals. We placed the TCD probe in the posterior portion of the cortex. This position was fixed in a stationary position throughout the protocol.

ELISA

Commercially available ELISA Kits were used to quantity CSF ERK MAPK (sham control, 1,4h post FPI) and adrenomedullin (sham control, 1h post FPI) (Assay Designs) concentration. Phosphorylated ERK MAPK enzyme values were normalized to total form and then expressed as percent of the control condition.

Statistical analysis

Pial artery diameter, CBF, and CSF ERK MAPK and adrenomedullin values were analyzed using ANOVA for repeated measures. If the value was significant, the data were then analyzed by Fishers protected least significant difference test. An α level of $p<0.05$ was considered significant in all statistical tests. Values are represented as mean ±SEM of the absolute value or as percentage changes from control value.

Results

PHE increases CPP in both sexes after FPI, but such increases in CPP are less in male compared to female piglets during combined hypotension and FPI

One hour after FPI, ICP was higher in the male than the female piglet at an equivalent insult levels (1.9 ± 0.1 atm) (Fig 1A). ICP values during combined moderate or severe hypotension and FPI were also greater in the male compared to the female (Fig 1A). PHE (1μg/Kg/min iv) pre-treatment (30 min before FPI) and post-treatment (30 min after FPI) blunted FPI associated increases in ICP at 1h post injury in male and female piglets (Fig 1A). During combined hypotension and FPI, PHE blunted further increases in ICP in the female, but not the male (Fig 1A). In contrast, PHE had no effect on elevated ICP in the male during combined hypotension and FPI. CPP was lower at 1h post injury more in the male than the female (Fig 1B). Similarly, during combined hypotension and FPI, CPP was also lower more in the male than the female (Fig 1B). PHE increased CPP more in the female than the male both after FPI and combined hypotension and FPI (Fig 1B). PHE produced comparable increases in MAP in male and female piglets (Fig1C). These data indicate that brain injury of equivalent intensity produces sex dependent differences in ICP and CPP. The mechanism by which PHE infusion preserves CPP in female piglets is via decreasing ICP and this effect is not observed in male piglets.

PHE prevents sex dependent reductions in pial artery diameter and CBF after FPI

FPI produced greater reductions in pial artery diameter and blood flow in the parietal cortex in male compared to female pigs (Fig 2,3), similar to previous observations (8,17). PHE pre and post-treatment blunted FPI-induced reductions in pial artery diameter and CBF in the female and male (Fig 2,3).

PHE prevents loss of CBF autoregulation during hypotension after FPI in the female but not the male piglet

CBF in the parietal cortex was unchanged during hypotension prior to FPI, supportive of intact cerebral autoregulation pre-insult (Fig 3). After FPI, CBF was lower in the male than in the female (Fig 3). CBF was reduced further during moderate and severe hypotension, but such reductions were greater in the male than the female (Fig 3). In contrast, PHE (pre-treatment) prevented loss of cerebral autoregulation during hypotension after FPI in the female (Fig 3). Similar protection of cerebral autoregulation in the female was observed with PHE administration 30 min after FPI (Fig 3). However, reductions in CBF during hypotension after FPI were unchanged in males with PHE pre- and post-treatment (Fig 3), indicating a complete lack of protection by PHE. These data indicate that PHE prevents loss of CBF autoregulation during hypotension after FPI in a sex dependent manner.

PHE prevents loss of pial artery dilation during hypotension after FPI in the female but aggravates such loss in the male piglet

Prior to FPI, hypotensive pial artery dilation was significantly less in male than female piglets (Fig 4), similar to that recently published (8,17). Within 1 hr of FPI, hypotensive pial artery dilation was impaired in both sexes, but the degree of impairment was greater in the male compared to the female (Fig 4). Treatment with PHE (1 μg/Kg/min iv) under sham control conditions had no effect on hypotensive pial artery dilation (Fig 4). However, pre- or post-treatment with PHE in the setting of FPI reversed hypotensive pial artery dilation to vasoconstriction in the male (Fig 4A). In contrast, pre-treatment with PHE had no effect on hypotensive pial artery dilation in the setting of FPI in the female, while PHE post-treatment partially prevented hypotensive dilation (Fig 4B). Papaverine induced pial artery dilation was unchanged by FPI and PHE (Fig 5). Further, pial artery dilation was unchanged by PHE in the absence of FPI (Fig 5).

PHE prevents reductions in ARI in the female, but increases such reductions in the male after FPI

Calculated autoregulatory index (ARI) was unchanged during moderate and severe hypotension prior to FPI in both sexes, but was reduced more in the male than the female after FPI (Fig 6). PHE pre-treatment fully had no effect on ARI after FPI in the female, while PHE post-treatment blunted change in the female (Fig 6). In contrast, PHE significantly further decreased ARI during moderate and severe hypotension compared to its absence in the male after FPI (Fig 6). These data indicate that PHE is protective of ARI after FPI in the female, but increases loss of ARI in the male post injury.

FPI produces phosphorylation of CSF ERK MAPK which is blunted by PHE in the female but increased by PHE in the male

FPI produced a marked phosphorylation of CSF ERK MAPK within 1h (Fig 7). ERK MAPK phosphorylation induced by FPI was markedly greater in the male than the female (Fig 7). PHE pre- or post-treatment blunted ERK MAPK phosphorylation after FPI in the female (Fig 7). In contrast, PHE pre- or post-treatment induced ERK MAPK upregulation in the male (Fig 7). U 0126 (1 mg/kg iv), a putative ERK MAPK antagonist, blocked ERK

MAPK upregulation in males and females after FPI (Fig 7), similar to prior studies (19) , supportive of its efficacy as an ERK MAPK antagonist.

CSF Adrenomedullin (ADM) is upregulated after FPI in the female and potentiated by PHE, unlike in the male where CSF ADM is unchanged after FPI and decreased in CSF by PHE

CSF adrenomedullin (ADM) is upregulated after FPI in the newborn female but unchanged in the newborn male (Fig 8), as previously reported (8). PHE (1 μg/Kg/min iv) pre- or post-treatment potentiated CSF ADM upregulation after FPI in the female (Fig 8). In contrast, there was a modest decrease in CSF ADM produced by PHE in the setting of FPI in the male (Fig 8).

Blood chemistry

There were no statistical differences in blood chemistry between sham control, FPI, and FPI drug treated animals before or after all experiments. The amplitude of the pressure pulse, used as an index of injury intensity, was equivalent in male and female FPI-vehicle and FPI-drug treated animals (1.9 ± 0.1 atm).

Discussion

Results of this study show that systemic MAP support with PHE robustly protects cerebral autoregulation in the setting of FPI in the female, but not the male newborn pig. In fact, the systemic vasopressor PHE worsens the loss of cerebral autoregulation after FPI in the male piglet, suggesting sex dependent differences in outcome when using the same clinically driven critical pathway for treatment of pTBI. FPI produces greater increases in ICP and reductions in parietal cortical blood flow and CPP in male compared to female newborn piglets, corroborating our recent studies (17). PHE blunted FPI associated increases in ICP post injury in male and female piglets. However, during combined hypotension and FPI, PHE only prevented further increases in ICP in the female, but not the male. PHE increased CPP more in the female than the male after FPI. These data indicate that FPI produces sex dependent differences in ICP and CPP, while PHE infusion has different efficacy in elevating CPP in male and female piglets. A caveat to data interpretation for the role of PHE, however, is that outcome may well depend on the observed innate sex dependent differential severity of injury. Nonetheless, the mechanism by which PHE infusion preserves CPP in female piglets is via decreasing ICP since CPP = MAP ICP. Since post-treatment with PHE largely prevents brain injury-induced impairment of cerebral autoregulation in the female piglet, these data provide support for use of this intervention in the critical care treatment of brain injured girls, but calls into question the merit of its use in brain injured boys. However, we do express caution in over-interpretation in that these are newborn pig data which may or may not be the same as in newborn and/or young children. Clinical studies will be needed to confirm these pre-clinical data. Since responses to papaverine were unchanged by PHE, these data indicate that impaired autoregulation is not an epiphenomenon and that use of this pressor does not have a pandemic effect on all vasodilator systems.

A second key new finding of this study is that PHE blocks ERK MAPK upregulation after FPI in the female, but not the male, where it actually aggravates such upregulation. Prior studies have shown that FPI associated upregulation of ERK MAPK contributes to reductions in CBF, impaired cerebral autoregulation during hypotension, and histopathology (17,19). Taken together, these data indicate that cell signaling should be equally considered as an important determinant of outcome, as is use of paradigms to increase CPP. These data further suggest that therapies for treatment of brain injured children should consider addition of interventions which block ERK MAPK, particularly in boys. Nonetheless, we do

acknowledge the caveat that sex dependent differences in ERK MAPK signaling may not be the only explanation for the observed innate differential sex based cerebral hemodynamic outcome after FPI.

Our prior studies have indicated a prominent role for impairment of K channel function and decreased production of CGRP peptides in cerebrovascular dysregulation during hypotension after FPI (7,8). Adrenomedullin (ADM) is a 52 amino acid peptide belonging to the CGRP family. In rats, adrenomedullin mRNA expression is upregulated after ischemia and may be cerebroprotective, particularly after stroke (22,23). ADM increases CBF and prevents ischemia after middle cerebral artery occlusion (24). Marked increases in CSF ADM in children occur after severe TBI and CBF was positively correlated with CSF ADM (25), suggesting that it may be neuroprotective and participate in the regulation of CBF after TBI (26,27). Our recent studies indicate ADM prevents sex dependent impairment of autoregulation during hypotension after FPI through inhibition of ERK MAPK upregulation (17). A final key observation in the present study is that PHE potentiated CSF ADM upregulation after FPI in the female. In contrast, there was a modest decrease in CSF ADM produced by PHE in the setting of FPI in the male. In the female, then, PHE simultaneously upregulates ADM and downregulates ERK MAPK to achieve better autoregulatory outcome after FPI, while in the male, the reverse happens. These data indicate that PHE may have multiple biochemical roles in addition to providing systemic pressor support to elevate CPP and thereby determine outcome after TBI. However, it is uncertain if other alpha adrenergic agonists will have effects similar to that of phenylephrine.

Hypotension and low CPP are associated with poor outcomes after TBI in children (28). Consequently, current 2003 Pediatric Guidelines recommend preventing or rapidly treating hypotension and recommend maintaining CPP above 40 mm Hg in children, noting that an age-related continuum for the optimal CPP likely lies between 40 and 65 mm Hg (29), with higher CPP values likely necessary for older children whose CPP needs may approximate those of adults (30). Despite these current therapeutic targets, there are no guidelines regarding how this should be achieved other than therapies to lower ICP by mannitol or hypertonic saline (31,32), the latter which may be desirable because of the added benefit of increasing CPP beyond what would be expected due to the drop in ICP (33). In addition to decreasing ICP, one commonly employed strategy to increase CPP is to use inotropes and or vasopressors to increase MAP. Although CPP is typically used clinically as a surrogate for CBF, ICP monitors are not universally used to guide CPP therapy, especially in young children (34). Thus, clinicians often rely on MAP to estimate cerebral perfusion. In the context of our present observations, arbitrarily setting MAP through PHE may not always achieve physiological conditions thought to be needed for a good outcome in brain injured children, particularly males.

In conclusion, results of this study indicate that elevation of CPP with PHE prevents impairment of cerebral autoregulation during hypotension after FPI through modulation of ERK MAPK upregulation in a sex dependent manner. These data suggest the role for sex dependent mechanisms in cerebral autoregulation after pediatric TBI.

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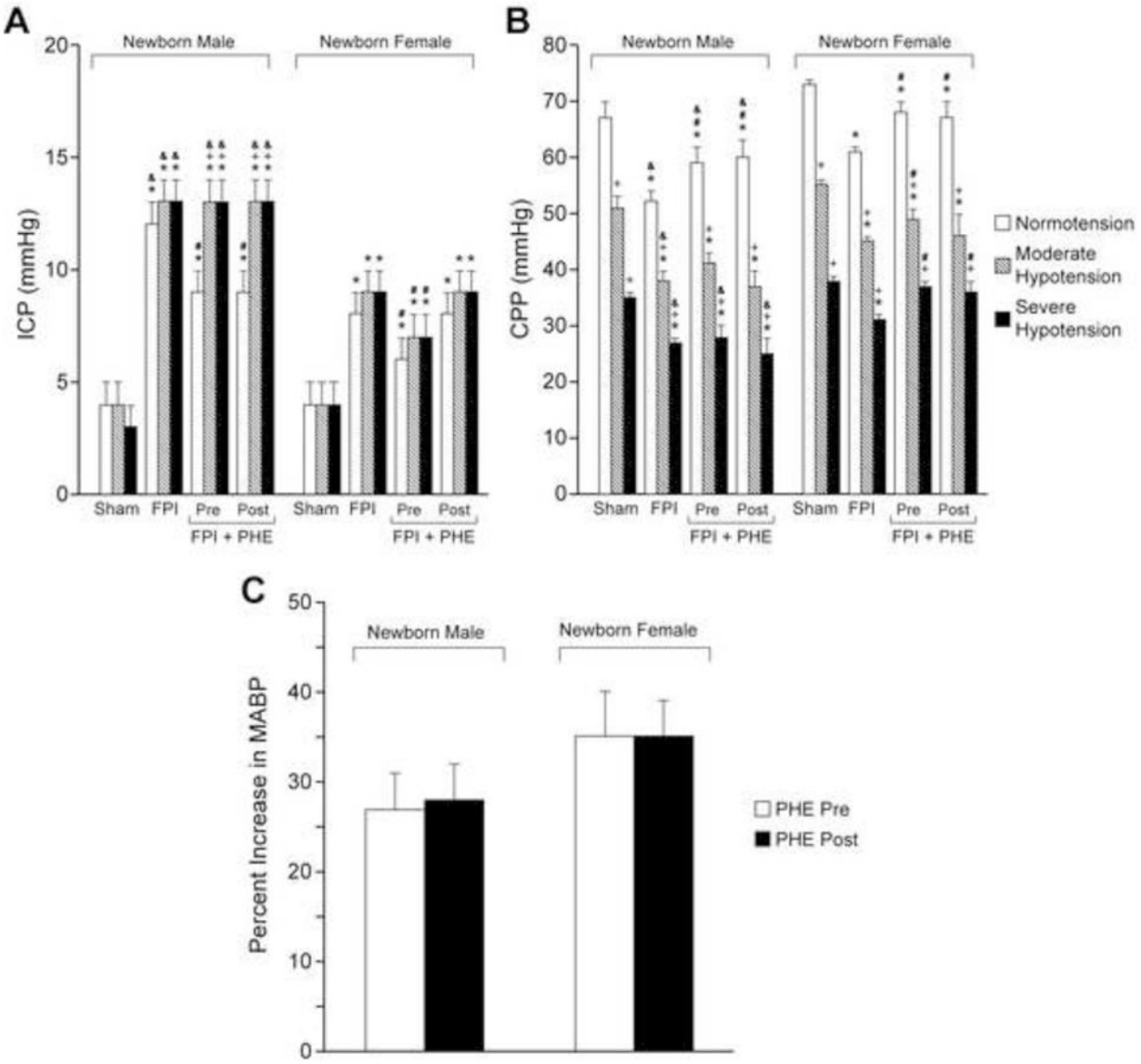

Figure 1.

A. ICP (mm Hg) and B. CPP (mm Hg) during normotension, moderate and severe hypotension in sham, FPI, and FPI + PHE (1 μg/Kg/min iv) pre-treatment and post-treatment (30 min before or 30 min after injury) male and female pigs, n=5. C. MAP (mm Hg) during PHE pre- and post-treatment in male and female pigs, n=5. $^{*}p<0.05$ compared with corresponding shame value. $^{+}p<0.05$ compared with corresponding normotension value $^{\#}p<0.05$ compared with corresponding nontreated FPI value $^{\&}p<0.05$ compared with corresponding female value.

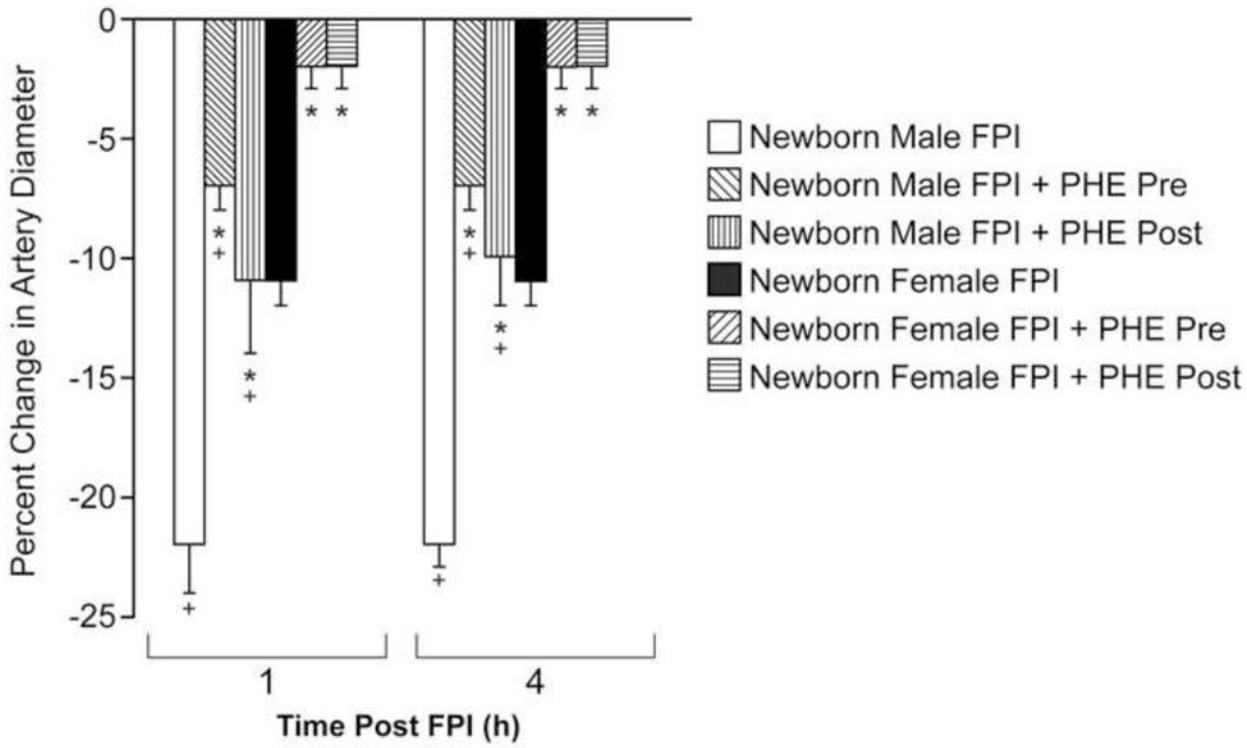

Figure 2.

Influence of FPI (h) on pial artery diameter in vehicle (FPI) and PHE (1 μg/Kg/min iv) 30 min pre or 30 min post FPI in newborn male and female pigs, n=5. $^{*}p<0.05$ compared with corresponding FPI vehicle value $^{+}p<0.05$ compared with corresponding female FPI value.

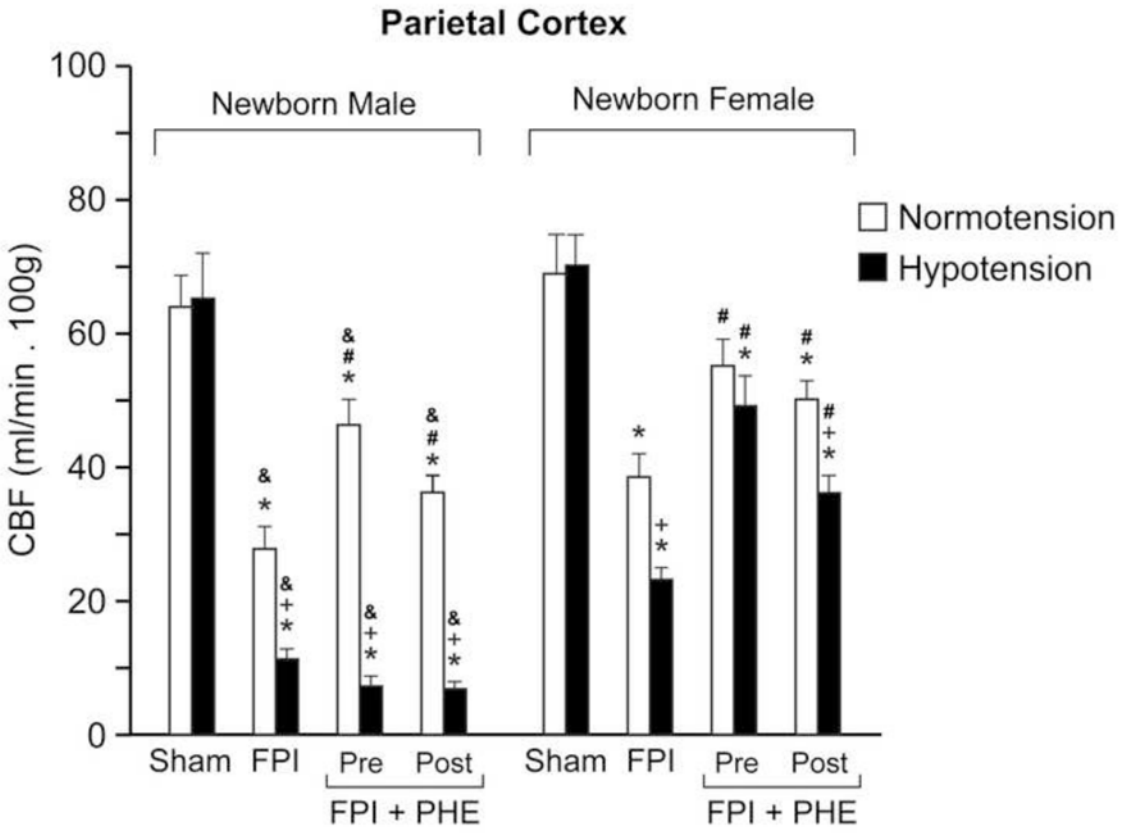

Figure 3.

CBF (ml/min .100g) in the parietal cortex during normotension and severe hypotension (hypotension) in sham, FPI, and FPI + PHE (1 μg/Kg/min iv) pre and post-treated newborn male and female pigs, n=5. $^{*}p<0.05$ compared with corresponding sham value $^{+}p<0.05$ compared with corresponding normotension value $^{\#}p<0.05$ compared with corresponding FPI non-treated value $^{\&}p<0.05$ compared with corresponding female value.

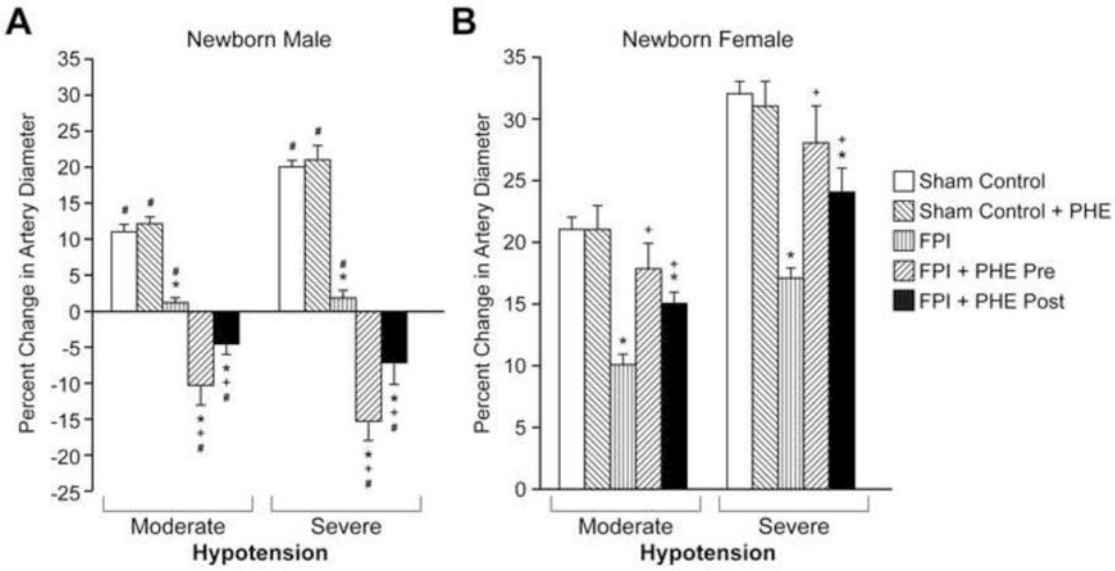

Figure 4.

Influence of moderate and severe hypotension on pial artery diameter in newborn male (A) and female (B) pigs before injury (sham control), before injury treated with PHE (1 μg/Kg/min iv), 1 h after FPI, 1h after FPI treated 30 min prior to injury with PHE, and 1h after FPI treated with PHE 30 min after injury, n=5. $^{*}p<0.05$ compared with corresponding sham value $^{+}p<0.05$ compared with corresponding FPI nontreated value $^{\#}p<0.05$ compared with corresponding female value.

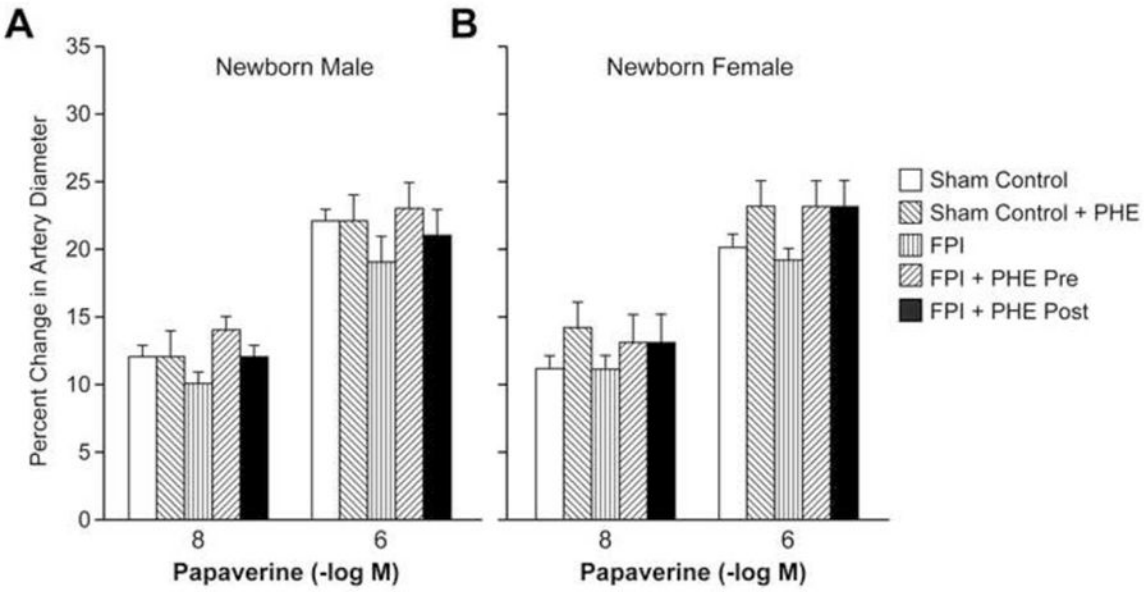

Figure 5.

Influence of papaverine (10^{-8}, 10^{-6} M) on pial artery diameter in newborn male and female pigs before injury (sham control), before injury treated with PHE (1 μg/Kg/min iv), 1 h after FPI, 1h after FPI treated 30 min prior to injury with PHE, and 1h after FPI treated with PHE 30 min after injury, n=5.

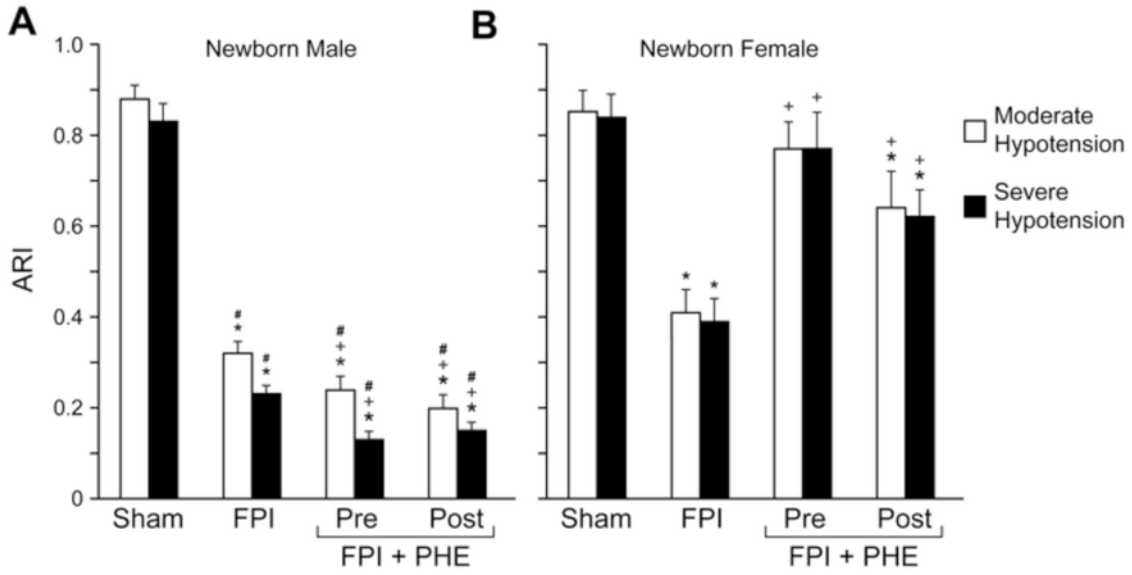

Figure 6.

ARI values for moderate and severe hypotension in sham, FPI, and FPI (1 μg/Kg/min iv) pre- or post-treated newborn male and female pigs, n=5. $^{*}p<0.05$ compared with corresponding sham value $^{+}p<0.05$ compared with corresponding FPI nontreated value $^{\#}p<0.05$ compared with corresponding female value.

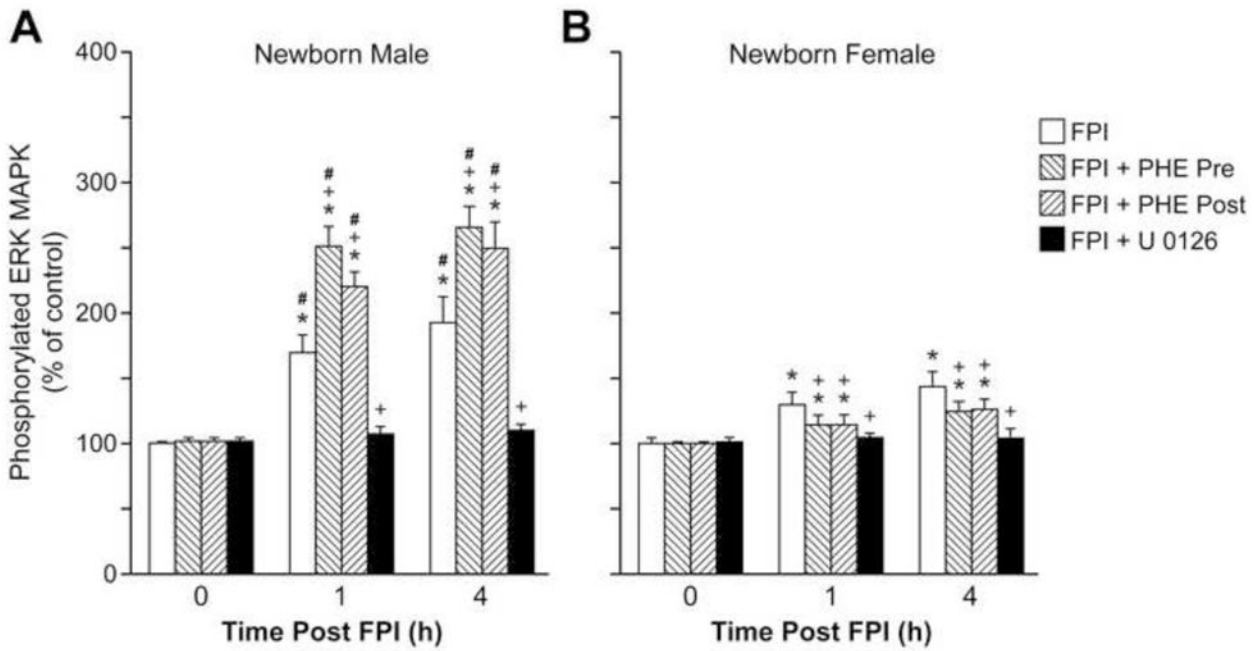

Figure 7.

Phosphorylation of ERK MAPK in CSF prior to FPI (0 time), as a function of time after FPI (h) in pigs treated with vehicle (FPI), PHE (1 μg/Kg/min iv) pre- or post-treatment (30 min) + FPI, or U 0126 (1 mg/kg iv) + FPI, n=5. Data expressed as percent of control by ELISA determination of phospho ERK MAPK and total ERK MAPK isoforms and subsequent normalization to total form. A: newborn male, B: newborn female. $^{*}p<0.05$ compared with corresponding 0 time value $^{+}$ $p<0.05$ compared with corresponding vehicle treated value $^{\#}p<0.05$ compared with corresponding female value.

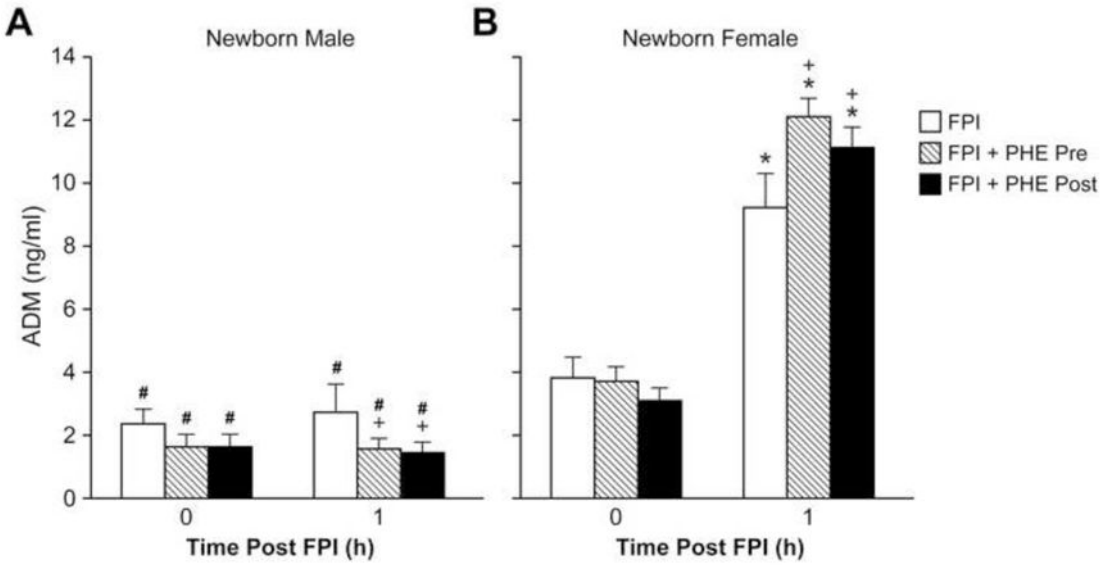

Figure 8.

ADM (ng/ml) in CSF prior to FPI (FPI (0 time), as a function of time after FPI (h) in pigs treated with vehicle (FPI), or PHE (1 μg/Kg/min iv) pre- or post-treatment (30 min) + FPI, n=5. A: newborn male, B: newborn female. $^{*}p<0.05$ compared with corresponding 0 time value $^{+}p<0.05$ compared with corresponding vehicle treated value $^{\#}p<0.05$ compared with corresponding female value.