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THE EFFECTS OF POSTNATAL ESTROGEN THERAPY ON BRAIN DEVELOPMENT IN PRETERM BABOONS

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Abstract

Objective—Estrogen receptors are present within the fetal brain suggesting that estrogens may exert an influence on cerebral development. Loss of placentally-derived estrogen in preterm birth may impair development.

Study Design—Baboons were delivered at 125 days of gestation (term~185 days), randomly allocated to receive estradiol (n=10) or placebo (n=8) and ventilated for 14 days. Brains were assessed for developmental and neuropathological parameters.

Results—Body and brain weights were not different between groups but the brain/body weight ratio was increased ($p<0.05$) in estradiol-treated animals. There were no differences ($p>0.05$) between groups in any neuropathological measure in either the forebrain or cerebellum. There were no intraventricular hemorrhages; one estradiol animal displayed ectatic vessels in the subarachnoid space.

Conclusions—Brief postnatal estradiol administration to primates does not pose an increased risk of injury or impaired brain development.

Keywords

Postnatal estradiol; premature delivery; brain injury; brain development; baboon

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INTRODUCTION

During human pregnancy, the placenta is the primary site of estrogen secretion, utilizing precursors that arise from both maternal and fetal compartments.¹ Fetal plasma estradiol (E₂) levels increase progressively during late gestation, rising further with the onset of parturition and falling in the early postnatal period due to the loss of the placentally-derived hormone.^{2, 3} Estrogen receptors are present in the primate lung⁴ and throughout the primate⁵ and human⁶ brain during fetal development suggesting that their activation by E₂ might play a role in the normal development of these organs, possibly as trophic factors.⁷ Indeed, it has been reported that in the brain, estrogen promotes axonal and dendritic growth and synapse formation⁸ and acts as a proliferative agent during critical stages of cerebral cortical development.⁹ In addition, E₂ protects the neonatal brain from hypoxia-ischemia.^{10, 11} Preterm infants commonly have low levels of estrogen and progesterone due to the lack of placental supply; it is possible that this impacts adversely on pulmonary and neural development and function.

In a primate model of bronchopulmonary dysplasia (BPD) postnatal E₂ treatment has beneficial effects on cardiovascular and pulmonary function and lowers the requirements for ventilatory support.⁴ Whether postnatal E₂ treatment affects the immature brain is unknown. Thus the aim of the present study was to evaluate the effects of postnatal E₂ administration on brain growth and the pattern of cerebral injury in prematurely delivered baboons cared for in a neonatal intensive care unit.

MATERIALS AND METHODS

Animal studies were performed at the Southwest Foundation for Biomedical Research in San Antonio, TX. Animal husbandry, handling and procedures conformed to American Association for Accreditation of Laboratory Animal Care guidelines.

Delivery and ventilatory management

Pregnant baboon dams (*Papio papio*) with timed gestations were treated with antenatal steroids before elective delivery at 125±2 days of gestation (dg, term 185 days).⁴ At birth animals were weighed, sedated, intubated and treated with 4ml/kg surfactant (Survanta, courtesy Ross Laboratories, Columbus, OH); ventilatory support was provided for 14 days. Animals were randomly assigned to either placebo (n=8) or estradiol (E₂, n=10) groups. Complete surgical procedures, animal care and ventilator management have been described previously.^{12–15}

Administration of E₂

Animals assigned to the E₂ group received a 0.5 mg, 21 day extended release pellet, placed subcutaneously in the left axilla at 1 hour of life; placebo animals received a control pellet. A second control or E₂ pellet was placed in the right axilla on day 7. The rationale for the dosing regimen has been described previously.⁴ Briefly, the dose was chosen to achieve E₂ levels that were in the upper range of the concentrations observed in the latter third trimester in fetal baboons. Serum E₂ levels, determined by radioimmunoassay, were measured in additional fetal baboons at 125dg, 140dg, 160dg and 180dg to determine normal fetal levels of E₂. Levels of E₂ were determined in placebo and E₂-treated animals at 6 hours of life and at 1, 2, 3, 7, 10, and 14 days.

Physiological data

PaO₂, PaCO₂, pH, fraction of inspired oxygen (FiO₂), systolic, diastolic and mean arterial blood pressure (BP), and heart rate were monitored continuously throughout the

experimental period. Oxygenation (OI) and ventilation (VI) indices were calculated.⁴ We also examined the relationship between the baboon's physiological instability and measurements of brain growth and injury. The "interval flux" of physiological variables was calculated as a surrogate measure of the physiological instability.¹⁶ We first determined the maximum and minimum values of each variable during a specified time interval; the interval flux was the difference between these values.¹⁶ For each animal we then: 1) identified the maximum flux; and 2) calculated the mean of the interval fluxes over the entire experimental time period. A greater degree of flux, particularly in FiO_2 , is associated with increased neuropathology.^{17–19}

Histological Analysis

Brains were weighed, immersed in 4% paraformaldehyde in 0.1M phosphate buffer and eleven blocks from the right forebrain (at 5mm intervals) and a mid-sagittal block from the cerebellar vermis of each brain were processed to paraffin. Ten (8 μ m) sections were collected from the rostral surface of each block of the forebrain and in the sagittal plane for cerebellar sections. A section from each block was stained with hematoxylin and eosin (H&E) and assessed for gross morphologic changes, including hemorrhages, lesions or infarcts, neuronal death, axonal injury and gliosis. Masson's trichrome was used to assess for collagen deposition and Perl's stain to visualize hemosiderin deposition, indicative of a bleed having occurred at least 48h prior to postmortem. Sections were scored for hemorrhages, infarcts and cystic lesions (0-absent; 1-present).

Immunohistochemistry for rabbit anti cow-glial fibrillary acid protein (GFAP, 1:500, code #20334, Sigma, St Louis, MO, USA) was used to identify astrocytes; rabbit anti-ionized calcium-binding adapter molecule 1 (Iba1, 1:1500, code #019-19741; Wako, Richmond, USA) to identify microglia/macrophages; mouse anti-human Ki67 clone MIB-1 (1:100; DakoCytomation, Glostrup, Denmark) to identify proliferating cells; mouse anti-chicken myelin basic protein (MBP, 1:100; Chemicon, USA) to assess the extent of myelination; rabbit anti-von Willebrand factor (1:800; Abcam, Cambridge, UK) to identify blood vessels; rabbit anti-caspase3 (1:500; Cell Signaling Technology, MA, USA) to identify cells undergoing cell death (apoptosis and necrosis), and rabbit anti-goat p27 cell cycle inhibitory marker (1:1000, Millipore, Billerica, MA USA), to identify post-mitotic cells, as described previously.^{16, 18}

All analyses were performed on all brains in the study and measurements made on coded slides blinded to the observer.

Quantitative Analysis: Forebrain

For each animal, all measurements were made on a section from each block, unless otherwise stated, using an image analysis system (Image Pro v4.1, Media Cybernetics, Maryland, USA). All values were calculated as mean of means for each group; measurements of cell numbers were expressed as cells/mm².

Volumetric measurements—Cross-sectional areas of regions in the right forebrain were assessed in H&E-stained sections using a digitizing tablet (Sigma Scan Pro 4, Media Cybernetics, California, USA); volumes of the white matter (WM), neocortex, deep grey matter (basal ganglia, thalamus and hippocampus) and ventricles were then estimated using the Cavalieri principle.²⁰

Surface folding index (SFI)—The SFI, which gives an estimation of the expansion of the surface area relative to volume, was determined.²¹

Areal density of astrocytes—GFAP-IR cells were counted ($\times 660$) in randomly selected areas (0.02mm^2) of the deep and subcortical WM, neocortex (3 sites in blocks from frontal/temporal, parietal/temporal and occipital lobes in layers 5 and 6) and hippocampus (stratum radiatum in the CA1 region).

Areal density of oligodendrocytes—MBP-IR oligodendrocytes were counted ($\times 300$) in 2 randomly selected areas (0.42mm^2) in both the deep and subcortical WM from the parietal/temporal lobe.

Areal density of microglia/macrophages—In Iba1-IR sections, cells were counted in randomly selected areas ($\times 660$; sample area 0.02mm^2) of both the deep and subcortical WM. In the neocortex (layers 2–6) a section from each lobe was selected and 3 regions (dorsal, lateral, ventral) sampled in each section ($\times 660$).

Percentage of white matter occupied by blood vessels—Point counting²¹ was performed in von Willebrand factor-IR sections to determine the density of blood vessel profiles in deep and subcortical WM and neocortex ($\times 660$) as an indicator of vasodilation or vasculogenesis.

Ki67-IR cells—In the neocortex, Ki67-IR cells were counted ($\times 660$) in the dorsal and ventral regions of the subventricular zone (sample area 0.02mm^2).

Activated caspase3-IR cells—Cells were counted ($\times 660$) in deep WM in the 5 fields (0.02mm^2) with the highest concentration of positively-stained cells.²²

GFAP-IR radial glial fibers—Sections from each lobe were scored for the presence of GFAP-IR fibers on a scale of 0–3 (0-none; 1-occasional; 2-moderate; 3-considerable).

Quantitative Analysis: Cerebellum

Sections were scored for hemorrhages and infarcts as for the forebrain.

The width of the external granule cell layer (EGL) and the molecular layer were assessed using the image analysis system as previously described.¹⁸

Ki67-IR cells—In 10, $75\mu\text{m}$ lengths of EGL ($\times 600$), the number of Ki67-IR cells was expressed as the proportion of total cells in the region. p27-IR staining was examined to determine the expression pattern in relation to Ki-67-IR. In the deep cerebellar WM, 5 regions were randomly sampled in 2 sections per animal ($\times 600$) and mean density of Ki67-IR cells determined.

Percentage of WM occupied by blood vessels—Point counting²¹ was performed in 2 regions of the deep WM ($\times 660$) from one von Willebrand-IR section from each animal.

Statistical Analysis

Linear regression analysis was carried out on data from the combined groups to determine if there was a correlation between: a) physiological variables (maximum and mean fluxes for pH, PaO_2 , PaCO_2 , FiO_2 , OI, VI and blood pressure and cardiac output) and quantitative variables (volumetric measurements, oligodendrocyte, astrocyte and microglial densities; and b) volumetric measurements and oligodendrocyte, astrocyte and microglial densities. Differences between parameters in E_2 -treated and placebo groups were tested using Student's t-tests; for all analyses a probability of $p < 0.05$ was considered to be significant.

RESULTS

Serum E₂ Levels

Serum E₂ levels have been reported in detail previously.⁴ Briefly, with E₂ administration, the initial levels were 1,000pg/ml at 6 hours of life, falling to 600pg/ml on day 2 and 230pg/ml by day 7. After insertion of a second E₂ pellet, levels rose to 400–500pg/ml. With the exception of day 7, levels were greater than in the placebo group and equated to the upper range of concentrations observed in the latter third trimester in baboon fetuses.⁴

Prematurely-Delivered Newborn Group Characteristics and Physiology

Birth weights, gestational age at delivery and ratio of males to females were similar in placebo and E₂-treated groups.⁴ As reported previously⁴ the mean systemic blood pressure of the estrogen-treated animals was higher ($p<0.05$) and the OI and VI lower ($p<0.05$), than in placebo-treated animals. In addition there was a decrease ($p<0.05$) in the mean interval flux of FiO₂ in E₂-treated compared to placebo animals consistent with improved respiratory function. There was no difference ($p>0.05$) in the mean interval flux of pH, PaO₂, PaCO₂, SaO₂, MAP or heart rate between groups during the 14-day study period (Table 1).

Brain Growth and Development

Body, brain and cerebellar weights were not different between groups; however brain-to-body weight ratio was increased in E₂ animals compared to placebo animals ($p<0.05$; Table 2). There was no difference in the total volume of the forebrain, WM, neocortical or deep grey matter or ventricular volumes between E₂ and placebo groups. Neither was there a difference between the groups in the ratios of WM, neocortical or deep grey matter or ventricular volumes to forebrain volume, the ratio of WM/neocortex, or in the overall SFI of the forebrain ($p>0.05$; Table 2).

Brain Injury, Forebrain

There was no evidence of cerebral infarction or intraventricular hemorrhage in any animal. In the subarachnoid space of a brain from an E₂-treated animal, there were at least two thrombosed ectatic vessels associated with a hemorrhage (Figure 1A, B). Material within the space stained positively for hemosiderin suggesting that the vessel damage was of at least several days standing.

Areal density of cellular markers—There was no difference between groups in the density of astrocytes, oligodendrocytes, microglia/macrophages, proliferating cells in the SVZ, or caspase3-IR cells in the areas measured ($p>0.05$; Table 3).

Percentage of white and grey matter occupied by blood vessels—This was not different between groups in either the subcortical or deep WM or grey matter ($p>0.05$; Table 3).

Radial Glia—Intensely stained GFAP-IR radial glial fibers were present at the ventricular surface and projecting into the deep WM in all animals; there was no difference between groups (1.6 ± 0.2 , placebo vs 1.7 ± 0.02 , E₂; $p>0.05$)

Brain Injury, Cerebellum

There was no evidence of infarction, hemorrhages or abnormal development in either group. No significant differences ($p>0.05$) were observed between groups in the widths of the EGL or molecular layer, the expression pattern, number or proportion of Ki67-IR cells/mm EGL,

the number of Ki67-IR cells in the deep WM, or the percentage of WM occupied by blood vessels ($p>0.05$; Table 4).

Relationship of brain growth and injury to brain volume and physiology

Overall there was a positive correlation between ventriculomegaly and the mean flux in: 1) pH ($r^2=0.23$; $p<0.04$); 2) PaCO_2 ($r^2=0.44$; $p<0.003$); and 3) SaO_2 ($r^2=0.48$; $p<0.002$) suggesting that physiological instability is associated with impaired brain growth. There were no other correlations between physiological variables and quantitative parameters.

There were positive correlations between the presence of radial glial fibers and 1) the density of astrocytes in the deep WM ($r^2=0.29$; $p<0.03$) and 2) ventriculomegaly ($r^2=0.33$; $p<0.01$). There were negative correlations between the presence of radial glial fibers and 1) the density of oligodendrocytes in the deep WM ($r^2=0.26$; $p<0.03$), 2) the total volume of WM ($r^2=0.36$; $p<0.01$) and 3) the SFI ($r^2=0.25$; $p<0.04$), indicating that increases in radial glia are associated with larger ventricles, more astrocytes, fewer oligodendrocytes in the deep WM and a reduction in the total WM volume and extent of cortical folding. There were negative correlations between the total volume of WM and the density of astrocytes in the deep ($r^2=0.24$; $p<0.04$) and subcortical ($r^2=0.30$; $p<0.02$) WM, indicating that increased gliosis is associated with a decreased WM growth.

COMMENT

This study has shown that postnatal E_2 administration to prematurely-delivered baboons does not pose an increased risk of brain injury or impairment in brain development. There was a small but significant increase in the brain-to-body weight ratio in E_2 -treated compared to placebo-treated animals, suggesting a very mild protective effect. The reduction in flux in physiological measures and improved respiratory function may be important as mechanisms for improved cerebral growth. We note however that physiological flux in the systemic circulation is often reflected in the cerebral circulation due to immature autoregulation²³ (and results in cerebral injury such as intraventricular hemorrhage). Thus in the present study there may be additional trophic factors underlying the improved cerebral growth.

In absolute terms, as we have reported in previous studies using this model,^{16–19, 24} premature delivery per se increased the incidence of subtle neuropathologies and reduced the normal trajectory of brain growth. For example the mean brain weight of normally grown gestational controls at ~140dg (coinciding with the end of the study period) is $60.0\pm1.6\text{g}$ ²⁴ compared to $45.8\pm1.2\text{g}$ for E_2 -treated and $44.3\pm0.9\text{g}$ for placebo-treated animals. There was no difference between E_2 - or placebo-treated animals in brain weight, gyral formation, SFI or relative growth of grey or white matter. However in E_2 -treated animals there was a trend for an increase in deep grey matter volume and cerebellar weight both of which could contribute to the increase in brain-to-body weight ratio. There was not a difference in astrocyte, oligodendrocyte or microglial densities in any of the regions of the forebrain suggesting that E_2 does not specifically cause astrogliosis, affect the oligodendrocyte lineage and myelination or have an impact on the cerebral inflammatory response when administered at ~26–28 weeks human gestation equivalent. We note that with E_2 treatment there was a trend for a reduction in apoptosis in the white matter and an increase in neurogenesis in the forebrain subventricular zone, both factors that could be considered beneficial to brain development.

As neither the neocortical volume nor the width of the cerebellar molecular layer differed significantly between groups it is likely that axonal and dendritic growth and synaptogenesis were not influenced markedly by estrogen supplementation; the study period coincided with

proliferation of neuronal processes in both regions. The effects of E₂ on synaptogenesis might be apparent at later stages in development when the process reaches a peak.

E₂ treatment did not result in angiogenesis or alter the developmental dismantling of the radial glial structure. Furthermore, no novel form of brain injury or alteration in brain development was observed with E₂ treatment. Alterations could be present at the level of brain microstructure, receptor expression or neurotransmitter levels which were outside the scope of this study.

There was no evidence that E₂ had a specific adverse influence on growth of the cerebellum or that it had induced overt damage or hemorrhage. The external granular layer, site of granule cell proliferation, was similar to the placebo-treated animal in terms of width and the proportion of cells undergoing division. In the WM there was no evidence of alteration in the density of proliferating cells, presumably glia, neither was there any indication of angiogenesis or vascular dilatation.

The only potential adverse finding with E₂ treatment was the presence of at least two ectactic vessels in the forebrain subarachnoid space of one animal. This could have occurred by chance and not as a result of treatment; however the only other vascular abnormality we have observed throughout our studies of prematurely-delivered baboons was in an animal exposed to inhaled nitric oxide²⁴ another agent which has an effect on the vasculature.

In translating our findings to the human preterm infant, we acknowledge that there are limitations in our study including the small number of animals and the relatively short duration of the study, precluding conclusions on long term outcomes. We note that preterm baboons were electively delivered without any pre-existing complications such as infection, hypoxemia or growth restriction. Postnatal E₂ supplementation has not been widely used as a therapy for human preterm infants but in small randomized controlled studies, Trotter and colleagues have administered it to extremely low birth weight infants and reported an improvement in bone mineralization,²⁵ a tendency for improved neurologic outcome²⁶ and a reduction in severe retinopathy of prematurity and BPD.²⁷ No adverse side effects have been reported. The beneficial effect of E₂ might be greater in the presence of progesterone, an important sex steroid in human fetal development; combined E₂ and progesterone therapy may have beneficial effects in embryonic lung cells.²⁸

Conclusions

Postnatal treatment of the baboon infant with E₂ did not specifically exacerbate or ameliorate the risk of brain injury and altered development, associated with premature birth. Thus as E₂ does not appear to specifically affect the developing brain at the level examined here, its use as an efficacious postnatal therapy to improve the structure and function of the lungs and other structures could be considered. Further research is required before it is clear whether treatment confers clinically significant benefits, or poses any risks to the preterm infant.²⁹

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Figure 1. Thrombosed ectatic vessel and associated hemorrhage in the subarachnoid space stained with A) Masson's trichrome; arrows indicates vessel wall and B) Perl's stain; arrows indicates hemosiderin deposits (suggestive of a bleed at least 48 hours before postmortem). Scale bar = 400 μ m

Table 1

Physiological parameters

Parameter	Placebo (n=8)	Estradiol (n=10)
pH	0.14±0.01	0.13±0.01
PaO ₂ (mm Hg)	31.2±2.3	28.5±1.6
PaCO ₂ (mm Hg)	21.2±1.8	18.3±1.4
FiO ₂	0.14±0.01 *	0.10±0.01
MAP	7.8±0.5	9.3±0.7
HR	14.9±3.2	16.5±1.5
SaO ₂	8.9±0.9	7.8±0.7

Values are mean interval flux ± SEM. FiO₂, fraction of inspired oxygen; MAP, mean arterial pressure; HR, heart rate.

*
p<0.05.

Table 2

Body and brain weights and cerebral volumetric measurements

Parameter	Placebo (n=8)	Estradiol (n=10)
Body weight at necropsy (g)	383±16	356±13
Total brain weight (g)	44.3±0.9	45.8±1.2
Cerebellum weight (g)	1.47±0.11	1.59±0.07
Brain/body weight, ratio	0.12±0.004	0.13±0.00*
Forebrain volume (mm ³)	15,518±858	16,074±632
White matter volume (mm ³)	6718±259	6753±214
Neocortical volume (mm ³)	7080±566	7500±414
Deep grey matter (basal ganglia, thalamus, hippocampus) volume (mm ³)	1492±102	1598±92
Ventricle volume (mm ³)	228±14	222±18
Ventricle/total volume (%)	1.5±0.1	1.4±0.1
White matter/total volume (%)	43.6±1.4	42.2±1.0
Neocortex/total volume (%)	45.3±1.3	46.5±1.1
Deep grey matter/total volume (%)	9.6±0.4	9.9±0.3
White matter/neocortex (ratio)	0.98±0.06	0.92±0.04
Surface Folding Index (SFI)	44.2±2.6	44.5±2.2

*
p<0.05 compared to placebo.

Values are mean ± SEM. All volume measurements were made on the right hemisphere

Table 3

Quantitative forebrain parameters

Parameter	Placebo (n=8)	Estradiol (n=10)
Astrocytes in: deep WM (cells/mm ²)	378±37	400±27
subcortical WM (cells/mm ²)	328±26	376±23
neocortex (cells/mm ²)	116±7	145±21
hippocampus (cells/mm ²)	193±22	254±21
MBP-IR oligodendrocytes in: deep WM (cells/mm ²)	89±27	126±32
subcortical WM (cells/mm ²)	32±10	37±8
Iba1-IR microglia/macrophages in: deep WM (cells/mm ²)	149±19	166±24
subcortical WM (cells/mm ²)	73±5	102±12
neocortex (cells/mm ²)	44±3	43±4
% of neuropil occupied by blood vessels in: deep WM	1.9±0.1	1.7±0.4
subcortical WM	1.8±0.1	1.9±0.4
neocortex	1.6±0.2	1.4±0.3
Ki67-IR cells (cells/mm ²) in subventricular zone	1357±619	1583±189
Caspase3-IR cells (cells/mm ²) in deep WM	78±14	65±9

Values are mean ± SEM. WM, white matter.

Table 4

Quantitative cerebellar parameters

Parameter	Placebo (n=8)	Estradiol (n=10)
Width of EGL (μm)	30 $\pm$ 1	33 $\pm$ 1
Width of ML (μm)	76 $\pm$ 6	74 $\pm$ 4
Ki67-IR cells in EGL (cells/mm)	333 $\pm$ 13	382 $\pm$ 29
Ki67-IR cells in EGL – (% , proportion of all cells)	55 $\pm$ 2	58 $\pm$ 1
Ki67-IR cells in deep WM (cells/mm ²)	380 $\pm$ 78	442 $\pm$ 77
% of WM occupied by blood vessels	1.9 $\pm$ 0.5	2.6 $\pm$ 0.4

Values are mean $\pm$ SEM. WM, white matter; EGL, external granule layer; ML, molecular layer.