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Dexmedetomidine reduces cranial temperature in hypothermic neonatal rats

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BACKGROUND: The α2-adrenergic agonist dexmedetomidine (DEX) is increasingly used for prolonged sedation of critically ill neonates, but there are currently no data evaluating possible consequences of prolonged neonatal DEX exposure. We evaluated the pharmacokinetics and histological consequences of neonatal DEX exposure.

METHODS: DEX was administered (s.c.) to naive (uninjured) neonatal Lewis rats to provide acute (25 µg/kg, ×1) or prolonged (25 µg/kg three times daily, ×2 or ×4 d) exposure. Therapeutic hypothermia was simulated using a water-cooled blanket. Cranial temperatures were measured using an infrared thermometer. DEX concentrations were measured by LC-MS in plasma and homogenized brainstem tissue for pharmacokinetic analysis. Cortex, cerebellum, and brainstem were evaluated for evidence of inflammation or injury.

RESULTS: Prolonged neonatal DEX exposure was not associated with renal or brain pathology or indices of gliosis, macrophage activation, or apoptosis in either hypothermic or control rats. Plasma and brain DEX concentrations were tightly correlated. DEX peaked within 15 min in brain and reduced cranial temperature from 32 to 30 °C within 30 min after injection in cooled rats.

CONCLUSION: Prolonged DEX treatment in neonatal rats was not associated with abnormal brain histology. These data provide reassuring preliminary results for using DEX with therapeutic hypothermia to treat near-term brain injury.

In critically ill neonates, pain-related stress affects developmental outcomes, so appropriate pain management is paramount (1). Opioid infusions, sometimes in combination with benzodiazepines, are commonly administered with the intent to decrease pain, agitation, and stress responses in neonates exposed to frequent procedures, during mechanical ventilation, or during therapeutic cooling for hypoxic–ischemic encephalopathy (HIE). However, opioid and benzodiazepine exposure may produce neuroapoptosis and neurodevelopmental abnormalities (2,3). To reduce opioid exposure, alternative pain medications are needed, but few of the analgesics used in adults have been tested in neonates. Dexmedetomidine (DEX) has recently become a popular analgesic used in adult critical care (4).

A selective α-2 adrenoceptor agonist, DEX produces analgesic and sedative effects that can synergize with other analgesics (5,6). In randomized studies of adults, DEX lowers dosing requirements for adjunctive sedatives, shortens hospital stay, decreases psychosis, and may improve posthospitalization memory and cognitive function (7–9). It is recommended for short-term use only, less than 48 h. Minimal efficacy, pharmacokinetic, and safety data exist for neonates. Despite limited testing and without current Food and Drug Administration approval, the off-label use of DEX in pediatric and neonatal intensive care units is increasing (10–12). Since DEX can provide sedation and prevent shivering, it is a viable alternative to opioids for neonatal intensive care units patients undergoing therapeutic hypothermia as treatment for HIE. Concern is warranted because, unlike acute use for postsurgical recovery, neonatal sedation is often continuous and prolonged. The possible consequences of prolonged neonatal DEX exposure are unknown.

To more thoroughly understand the potential consequences of off-label DEX use in neonates, we conducted animal experiments to determine plasma and brain pharmacokinetics of DEX in neonatal rats. We evaluated pharmacokinetics and physiologic effects of DEX using both control and hypothermic animals. To evaluate safety, we examined both brain and kidney histopathology, including immunohistochemical markers of gliosis and apoptosis.

RESULTS

Clinical Use of DEX in Neonates

A total of 270 infants (<1 y old) were prescribed DEX at Seattle Children's Hospital from June 2013 to June 2014. **Figure 1** plots a frequency distribution to illustrate how many infants received prolonged (>36 h) DEX treatment (panel a) and mean duration of DEX exposure (panel b) for groups of infants separated by age at first dosing. These data illustrate that the current off-label DEX therapy for infants involves prolonged exposure lasting on average 2–3 wk.

Hypothermia and DEX in Rat Pups

Preliminary testing on a subset of $N = 10$ rats was performed to determine a suitable sedating dose. When placed supine,

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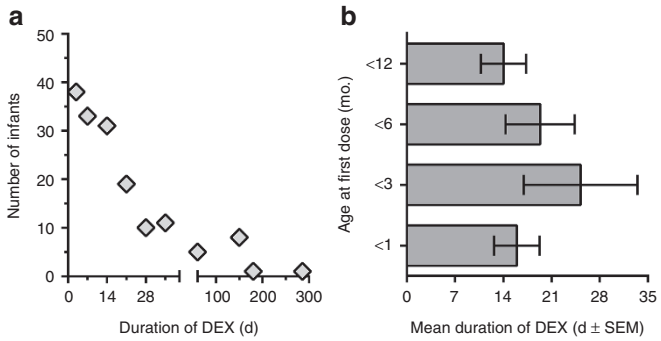

Figure 1. Duration (days) of prolonged (>36 h) dexmedetomidine (DEX) sedation for 157 infants (<1 y) undergoing intensive care at Seattle Children's Hospital (from June 2013 to June 2014). The left-sided panel (**a**) contains counts (filled diamond) of the total number of infants (y-axis) according to their duration of DEX treatment (x-axis) to illustrate the frequency distribution of prolonged DEX sedation. The right-sided panel (**b**) separates those data into 4 groups based on the age of infants at first treatment (< 1 mo. $N = 48$, 1–3 mo. $N = 39$, 3–6 mo. $N = 42$ and 6–12 mo. $N = 28$) to plot the corresponding mean (+SEM) duration of chronic DEX exposure. Not shown are data from infants given acute DEX therapy for less than 36 h ($N = 113$).

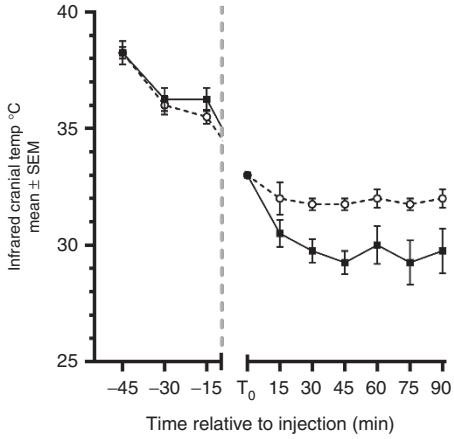

Figure 2. Neonatal rat cranial temperatures for untreated control rats (open circle, dotted line), and rats treated with 25 μg/kg dexmedetomidine (DEX) (black square, solid line) at time zero (T_0). P11 animals were removed from the dam and isolated for 45 min, then placed on a cooling blanket to initiate hypothermia (vertical dotted line) for 15 min prior to DEX. Recordings were made initially and every 15 min thereafter using a noninvasive infrared thermometer. Repeated measures ANOVA identified a time × treatment interaction ($F_{1,6} = 22.7$, $P \leq 0.01$) and *post hoc* tests found that DEX significantly decreased temperatures compared to control from 30 to 90 min postinjection ($P \leq 0.05$, $N = 4$/group).

100% of untreated P7 rats were able to right themselves to a prone position within 2 s of release. When tested at 15 and 30 min after treatment with DEX, 100% of rats given 10 μg/kg DEX were still able to right themselves within 2 s. In contrast, only 33% ($P = 0.076$ Fisher's exact test) of rats given 25 μg/kg DEX were still able to right themselves at 30 min. Righting reflex was restored in all rats when tested at 45 min after DEX treatment. Therefore, 25 μg/kg DEX treatments were used for all subsequent experiments. Because therapeutic hypothermia for HIE is used exclusively for term infants, a subset of eight P11 animals was used to measure the effect of hypothermia on head temperature with and without DEX treatment. **Figure 2** illustrates that when neonatal rats are placed on a cooling blanket (set to 24.6 °C), their head temperatures rapidly decrease to match a hypothermic criterion (32 °C), and head temperatures are further decreased by ~2 °C in rats injected with DEX at time zero (T_0).

Plasma and Brain Pharmacokinetics

Figure 3 compares the time course of plasma and brain DEX concentrations for control and hypothermic neonatal rats, and shows the corresponding correlations between brain and plasma DEX concentrations in the inset. Uptake of DEX into the neonatal rat brain was rapid, and equilibration between brain and plasma was apparent by 15 min after DEX injection (i.e., a parallel decline in plasma and brain DEX as illustrated by the tight correlation). A semilogarithmic plot (not shown) of the plasma data revealed a biexponential decline that was more pronounced for the hypothermia group. The mean (± SD) pharmacokinetic parameters of DEX for the control and hypothermia groups are presented in **Table 1**. The parameter estimates in **Table 1** did not differ significantly between the two treatment groups in that their 95% confidence interval overlapped. The initial distribution volume (33.0 vs. 13.7), terminal elimination half-life (88.8 vs. 139 min), mean residence time (120 vs. 192 min), and plasma clearance (0.345 vs. 0.259 ml/min/10 g weight) were all marginally nonsignificant, which raise the possibility that we may be limited by the small sample sizes ($N = 3$–5/time point) and had insufficient power to detect modest differences. It is noteworthy that the brain-to-plasma partition ratio of DEX was nearly identical in the two treatment groups, indicating that hypothermia did not alter the penetration of DEX across the neonatal blood–brain barrier. Lastly, two-way ANOVA comparison of the plasma and brain data confirmed significant effects of time, and suggested a trend for the time by hypothermia interaction ($P = 0.09$).

Tissue Histology and Immunohistochemistry

Brain and kidney tissues from neonatal rats exposed to either 2 or 4 d of repeated DEX injection and from hypothermic rats given a single injection were evaluated. H&E-stained transverse sections of cerebrum, brainstem, and cerebellum from each animal showed normal anatomy with no signs of neural degeneration, inflammation, or cytological atypia (**Figure 4**). Similarly, immunostained sections did not show any generalized or focal changes in the density or distribution of CD68-positive activated microglia, glial fibrillary acidic protein-positive fibrillary astrocytes (gliosis), or Caspase 3-positive cells (**Figure 4**). The density of activated microglia and Caspase 3-positive cells was extremely low (<1% of cells in most areas) and slightly higher in large white matter tracts of both experimental and control animals. Glial fibrillary acidic protein-positive astrocytes were concentrated in bilaterally symmetric zones around large white matter tracts, in portions of the subpial parenchyma, and in some discrete foci at the floor of the fourth ventricle. No differences in immunostaining were apparent when samples from the control and various experimental groups were compared (**Figure 4** and data not

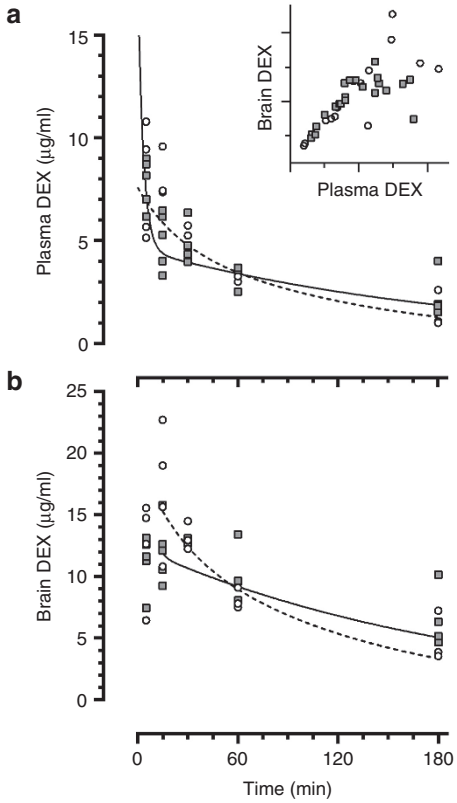

Figure 3. Pharmacokinetic plot of dexmedetomidine (DEX) concentrations measured in plasma (**a**) and in homogenized brain tissue (**b**) from individual neonatal rats. Neonatal rats were injected with 25 μg/kg DEX s.c. and euthanized at scheduled times. Data from P7 control rats (open circle) and P11 rats made hypothermic by exposure to a cooling blanket (filled square) are compared. The data are fit with biexponential regressions for both control (dotted line) and hypothermic rats (solid line). The inset (panel **a**) shows strong correlations between brain and plasma DEX concentrations for control ($R = 0.78$, $P \leq 0.001$) and hypothermic rats ($R = 0.64$, $P \leq 0.01$).

shown). Similarly, no renal histopathology was observed in any of the examined H&E-stained sections.

DISCUSSION

These experiments were conducted to describe the pharmacokinetics of DEX in plasma and brain tissue of both control and hypothermic neonatal rats, and to evaluate whether prolonged DEX treatment is safe. The principal observations were that DEX exposure in neonatal rats appears safe based on brain and kidney histology studies, since there were no indications of histopathology for forebrain, brainstem, cerebellar, and kidney tissues in either cooled animals given acute DEX, or control animals given repeated DEX for 2 or 4 d. By directly measuring DEX concentrations in brain tissue, we observed that DEX penetration into brain corresponds well with the reduction of cranial temperatures in neonatal rats made hypothermic by placement on a cooling blanket. In addition, our prescription data from a single-center confirmed that DEX is currently being used off-label for prolonged sedation of hospitalized infants requiring intensive care. These data provide preliminary information about the possible effects of DEX when

Table 1. Mean (± SD) DEX pharmacokinetic parameter estimates for control and hypothermic neonatal rats

Parameter, unit	Control	Hypothermia
A_1, μg/ml	2.35 ± 1.48	13.6 ± 7.4
λ_1, min^{-1}	0.0433 ± 0.0500	0.307 ± 0.092
A_2, μg/ml	5.23 ± 1.22	4.59 ± 0.36
λ_2, min^{-1}	0.00780 ± 0.00139	0.00498 ± 0.00057
$T_{1/2,1}$, min	16.0 ± 18.5	2.25 ± 0.68
$T_{1/2,2}$, min	88.8 ± 15.8	139 ± 16
Mean residence time, min	120 ± 15	192 ± 22
V_0, ml/10 g bwt	33.0 ± 5.6	13.7 ± 5.6
V_{ss}, ml/10 g bwt	41.5 ± 4.3	49.7 ± 3.8
CL, ml/min/10 g bwt	0.345 ± 0.031	0.259 ± 0.022
K_{br}	2.60 ± 0.28	2.83 ± 0.23

Neonatal rat pups were injected with 25 μg/kg s.c. DEX and then killed at scheduled times (range: 5–180 min).
DEX, dexmedetomidine.

administered to infants undergoing therapeutic hypothermia as treatment for HIE.

DEX is an α-2 adrenergic agonist and, in neurons, stimulates presynaptic α-2 adrenoceptor to inhibit release of monoamines (norepinephrine, serotonin, and dopamine); that effect is abolished by transgenic knockout of the α-2 receptor (13). In humans, α-2 receptors are concentrated in the nucleus of the solitary tract, dorsal motor nucleus of the vagus nerve, locus coeruleus, and reticular formation; so, the sedative effects of DEX likely involve action at these loci (14). For these reasons, we selected neonatal rat brainstem to profile the penetration of DEX into brain tissue and also examined these nuclei in the neuropathology studies.

Because there are reported neuroprotective effects of DEX treatment, DEX may be particularly beneficial for infants at risk for perinatal brain injury, who also require sedation during critical care. *In vitro* and *in vivo* experimental models of adult (15,16) and perinatal brain injury (17–20) have identified neuroprotective effects of DEX that are α-2A-dependent (19). For example, in adult rats given unilateral transient forebrain ischemia, the combination of DEX and hypothermia improved the short-term neurologic outcome compared to controls (21). The currently proposed protective mechanism involves promotion of brain-derived neurotrophic factor expression (17). Given the reports demonstrating DEX-associated neuroprotection, we expect that off-label DEX will become increasingly used when infants with HIE are undergoing therapeutic hypothermia. Therefore, it was important to identify plasma and brain DEX pharmacokinetics under hypothermic conditions compared to control conditions, to assess the effects of DEX treatment plus hypothermia on brain histology.

In the present study, DEX treatment rapidly decreased cranial temperature by 2 °C in cooled neonatal rats. Although α-2 receptor agonists, including DEX, are known to reduce core temperature in adult mice and rats (22), to our knowledge, the DEX-associated decrease in neonatal cranial temperature during hypothermia has not been previously described in animals

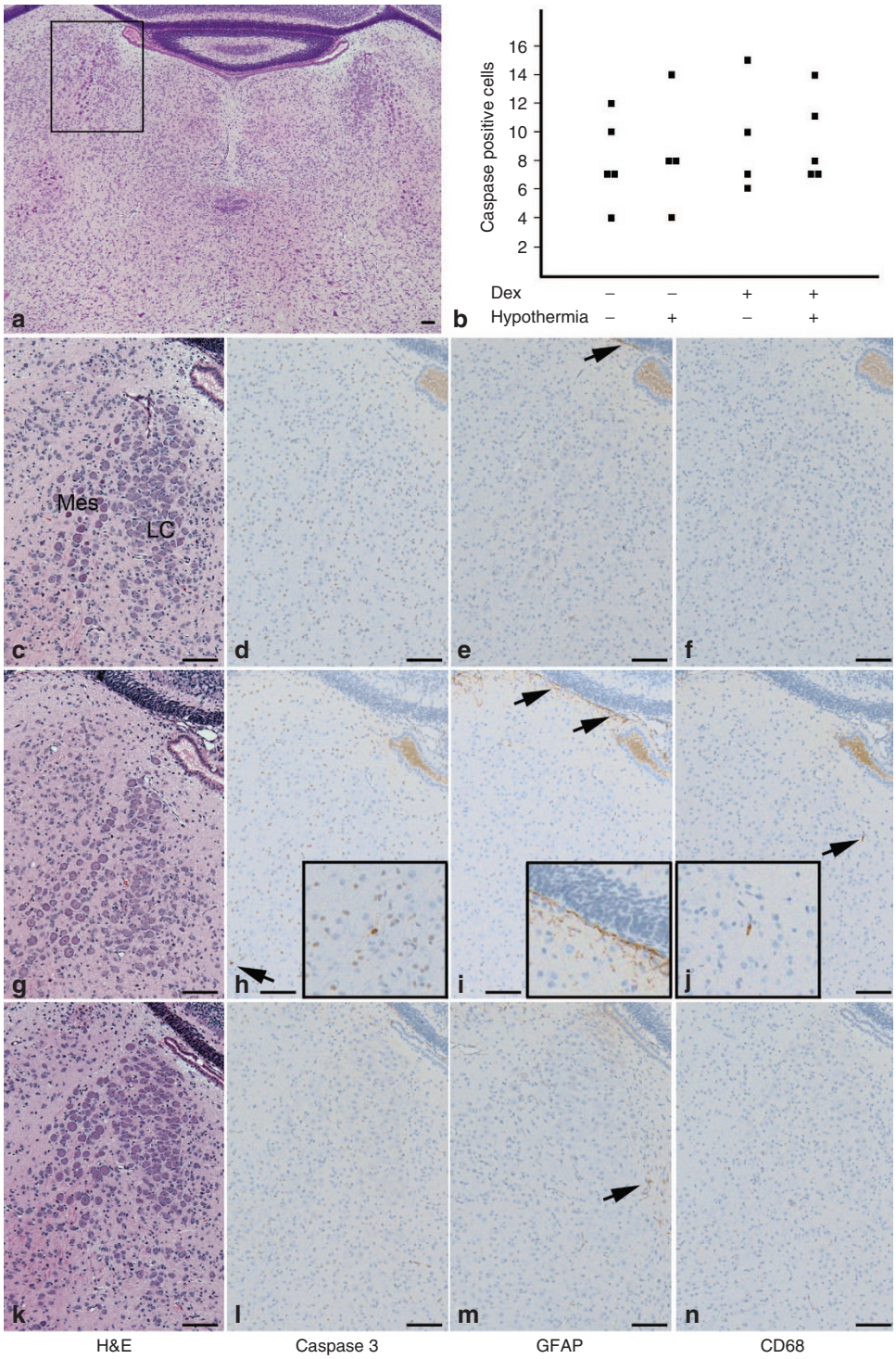

Figure 4. Rat brain and brainstems were examined using both histological and immunohistochemical indices but there were no differences between treatment groups. Panels show representative images of hematoxylin and eosin (H&E)-stained or immunostained brainstem tissue. Panel **a** is a low-magnification transverse section through the pontomedullary segment of brainstem from a saline-treated nonhypothermia control. Panel **b** plots the corresponding number of Caspase 3-immunopositive brainstem cells in treatment groups, and the x-axis uses plus and minus signs to indicate whether rats received dexmedetomidine (DEX) or hypothermia. Subsequent panels **c–n** are higher magnification images from the region highlighted in Panel **a** containing the mesencephalic nucleus (Mes) and locus coeruleus (LC) and adjacent sections were visualized with H&E stain (**c,g,k**) or immunostained for apoptosis using Caspase 3 (**d,h,l**), for astrocytes using glial fibrillary acidic protein (**e,i,m**), or for microglia using CD68 (**f,j,n**). Overall, immunoreactivity was sparse and a few immunopositive cells are indicated by arrows and enlarged in insets. Scale bars = 100 μm.

or humans. In anesthetized adult rats, α2-adrenergic agonists inhibit brown adipose and shivering thermogenesis via action at medullary premotor neurons in the rostral raphe pallidus (23). Future experiments in neonatal rats are warranted to examine if this observed temperature effect under hypothermic conditions is due to DEX-induced suppression of shivering,

increased cutaneous vasodilation, inhibition of brown adipose tissue thermogenesis, or disruption of hormonal thermoregulatory pathways. Alternatively, regarding the safety of neonatal whole-body cooling, it may be important to know how precisely autoregulating cooling devices detect and adjust for small drug-mediated changes in body temperature.

The rapid effect of DEX on cranial temperature corresponded precisely with the rapid uptake of DEX in brain tissue. DEX equilibrated rapidly between plasma and brain as indicated by the strong correlation between their concentrations post uptake. Comparisons of the pharmacokinetic parameters between control and hypothermic animals showed overlapping confidence intervals, although it is noteworthy that key parameters (initial distribution volume, elimination half-life, mean residence time, and plasma clearance) were only slightly overlapping. Ezzati *et al.* (24) recently reported that DEX clearance was reduced ~10-fold compared with adult values in the newborn piglet asphyxia model following hypoxic–ischemic brain injury and subsequent therapeutic hypothermia. Moving forward, it will be valuable to determine if hypothermia alters plasma clearance of DEX in human neonates.

We found no histological evidence of pathology in kidney or brain tissue for neonatal rats treated with repeated DEX for either 2 or 4 d at the dose tested. We examined the effects of DEX on renal histology because kidney expresses α-2 adrenoceptors (25) and DEX is excreted in urine. There was no evidence of renal histopathology for either control or hypothermic neonatal rats treated with DEX. Similarly, in brain tissue, there was no immunohistochemical evidence that repeated neonatal DEX exposure altered microglia or astrocyte activity, apoptosis, or anatomy. Although acute DEX provides protection against ischemic renal injury in adult rats (26) and improves renal function in adult dogs (27), it was not known whether repeated neonatal DEX exposure may be deleterious to kidney or brain. Given that prolonged off-label DEX use in infants is occurring, these data may be encouraging.

Our study has some limitations. Caution is always warranted when extrapolating information obtained from rats to humans. Nevertheless, this study is the first to consider possible pathophysiological effects of prolonged DEX exposure on neonatal mammalian brain. Species-specific responses to the DEX dose, duration, and timing of exposure may influence potential short- and long-term effects of DEX on the brain. So, although we saw no abnormal histopathologic effects of DEX at the dose tested, we cannot disqualify the possibility that there may be long-term deleterious effects of DEX on brain development, cognition, and behavior. We selected a sedating dose of DEX and estimated plasma and brain DEX pharmacokinetic parameters and correlations to inform the histological analysis. We did not investigate neuroprotective effects of DEX. We evaluated P7 control rats to model DEX sedation in premature infants, and hypothermia was created in P11 rats to model near-term treatment of HIE.

Proper management of neonatal pain using medications that are safe and effective remains a top priority. The challenge is to minimize pain for vulnerable preterm infants while simultaneously avoiding adverse effects of sedatives on neurobehavioral, motor, emotional, and cognitive developmental outcomes. Caution is increased for preterm infants because their neurodevelopment may be compromised by inappropriate exposure to either stress or drugs. For example, in preterm infants born < 32 wk gestation, the degree of neonatal pain and stress correlated with cortical thickness at 7 y of age, raising concerns about the long-term negative effects of early pain exposure (28). The increasing use of off-label DEX for neonatal sedation compels examination of the consequences of prolonged early DEX exposure. Additional studies should evaluate more long-term neurodevelopmental consequences of prolonged neonatal DEX exposure to corroborate the safety of this increasingly popular sedative.

METHODS

Clinical Use of DEX in Neonates

To evaluate the duration of clinical use of off-label DEX for neonatal sedation, we obtained approval to perform a focused IRB-exempt retrospective query to collect deidentified data describing the duration of DEX treatment for infants (<1 y old) at Seattle Children's Hospital from June 2013 to June 2014. Other than age and treatment duration, no other clinical data were obtained.

Animals

All experimental protocols followed US NIH guidelines and were approved by the Animal Care and Use Committees at the University of Washington. Time-mated pregnant Lewis rats were purchased from Harlan (San Diego, CA) and housed in the local vivarium under specific pathogen-free conditions with a 12-h light/dark cycle and free access to food and water.

Animal Protocol

Dexmedetomidine (Precedex, Hospira) was purchased in a 0.1 mg/ml concentration and diluted in saline to obtain a 5 μg/ml injectable solution. Subcutaneous (s.c.) injection volumes were adjusted to deliver the appropriate dose based on average daily body weights of rat pups. Sex was not a criterion and pups of both sexes were used. Preliminary tests to select a suitable sedative dose were performed by injecting postnatal day 7 (P7) rats with DEX doses ranging from 0.1 to 50 μg/kg, then testing their righting reflex (place the animal supine and record the time to reorient prone, 30 s max) every 5 min for 15 min. To determine the combined effects of cooling and DEX on cranial temperature, a noninvasive infrared thermometer was apposed directly to the side of the P11 rats' heads to record whole head temperatures. Cranial temperatures only were recorded every 15 min before and after placement on a 26.4 °C cooling blanket with or without combined DEX injection. To determine pharmacokinetics of DEX, a separate set of P7 rat pups was injected at time-zero; then rats were euthanized at 5, 15, 30, 60, or 180 min postinjection, and blood was collected directly from the heart, spun at 1000 ×g 10 min, and plasma was frozen at −80 °C. In addition, brainstem tissue was dissected, placed in microtubes, snap frozen in liquid N_2, and stored at −80 °C until assay. To evaluate histological effects of repeated DEX treatment (3×/day), rat pups were treated daily for either 2 or 4 consecutive days beginning at P7 and sacrificed at P12. Each day, three s.c. injections of DEX or saline (control) were given at about 09:00, 12:00, and 15:00 h. A separate set of P11 rats were injected with DEX, then placed on a cooling blanket for 4 h, injected with DEX again at 3 h, and sacrificed at P12 for histologic evaluation. All animals were euthanized by an overdose of Beuthanasia-D solution (2 ml/kg i.p. = 780 mg/kg). To collect organ tissues, euthanized animals underwent transcardial perfusion with buffered 4% paraformaldehyde fixative. Brain and kidney tissues were immersion fixed for 72 h and then processed for embedding in paraffin.

Dexmedetomidine Assay

Plasma samples (10–50 μl) were mixed with an internal standard (100 pg medetomidine-d3) in 30 mmol/l pH 8.9 borate buffer. Standards

were prepared using DEX-spiked plasma. Sample aliquots were transferred to clean solid-phase extraction tubes, and sequentially washed with H_20, 10 mmol/l pH 4 ammonium acetate, and methanol, then vacuum dried and analytes were eluted from solid-phase extraction tubes with 80:20:2 methylene chloride:isopropanol:ammonium hydroxide. Frozen brain tissue samples (25 mg) were spiked with 100 pg medetomidine-d3, homogenized in 0.1 N NaOH, and extracted twice with 3 ml of 98.5:1.5 heptane:isoamyl alcohol. The analytes were back extracted from the pooled organic phase into 2 ml of 0.05 mol/l sulfuric acid. The sulfuric acid phase was neutralized with 0.2 ml of ammonium hydroxide and extracted with 2 ml of 98.5:1.5 heptane:isoamyl alcohol. The organic extract was decanted. Plasma sample eluates and brain extracts were both evaporated at 40 °C and reconstituted in 1% formic acid in H_20 prior to analysis by liquid chromatography-mass spectrometry (LC-MS). The LC-MS consisted of an Agilent Technologies (AT, Santa Clara, CA) 1290 series UPLC and a Restek (Bellefonte, PA) Ultra Aqueous C18 column coupled to an AT G6410B mass spectrometer; system operation and data output were controlled by AT Mass Hunter software (version B.04). Calibration curves were constructed using peak height response ratio and were fit with a 1/×-weighted quadratic regression. Concurrent quality controls established that the lower limit of detection was 5 pg with an accuracy of 95 ± 3% (S/N 3:1) and an inter-run coefficient of variation of < 7%.

Pharmacokinetic Analysis

Preliminary analysis indicated that the plasma and brain concentration data for the control and hypothermia treatment groups followed a biexponential pattern of decline over time on a semilogarithmic plot. The pooled plasma concentration–time data for each treatment group were fitted to a biexponential equation: $A_1 \cdot exp(-\lambda_1 \cdot t) + A_2 \cdot exp(-\lambda_2 \cdot t)$, where λ_1 and λ_2 are the respective rate constants (min^{-1}) and *A1* and *A2* are the respective coefficients (μg/ml) of the exponential terms for the initial distribution and terminal elimination phase. The corresponding brain concentration–time data were also included in the regression fit by recognizing that brain concentration is in dynamic equilibrium with plasma concentration by 15 min after DEX injection; i.e., in the postuptake phase brain concentration is related to plasma concentration by a constant brain-to-plasma partition ratio (K_{br}). The nonlinear regression analysis was accomplished using the numerical module of the modeling software SAAM II v. 2.3 (The Epsilon Group, Charlottesville, VA). Mean, SD, and 95% confidence interval were estimated for the biexponential parameters, and the following derived parameters: half-life for the distribution and elimination phase ($T_{1/2,1}$, $T_{1/2,2}$), mean residence time, initial and steady-state volume of distribution (V_0, V_{ss}), and plasma clearance (CL). Significant difference in the parameter estimates between the control and hypothermia group was indicated by nonoverlapping 95% confidence intervals.

Histology and Immunohistochemistry

Transverse sections of cerebrum, brainstem, and cerebellum were embedded in paraffin and serial 4-μm sections were collected for hematoxylin and eosin (H&E) staining with adjacent sections used for immunohistochemistry. An automated immunostainer (Benchmark Ultra, Ventana, Tuscon, AZ), was used to perform immunohistochemistry. The following primary antibodies (species, dilution, incubation time; manufacturer) were used: anti-CD68 (mouse, 1:200, 32 min; Dako, Carpenteria, CA), anti-Caspase 3 (rabbit, 1:250, 32 min; Cell Signaling Technology, Danvers, MA), and anti-glial fibrillary acidic protein (mouse, 1:400, 16 min; Dako). The slides were examined and scored by a board certified pediatric pathologist (R.P.K.) who was blind to dosing conditions. H&E-stained kidney sections were also evaluated. Caspase 3-immunoreactive cells in transverse sections of the brainstem, at the pontomedullary junction excluding the cerebellum, were counted, other assessments of Caspase 3-, glial fibrillary acidic protein-, and CD68-immunoreactive cell densities were made subjectively.

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