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# Spinal Anesthesia in Infant Rats: Development of a Model and Assessment of Neurological Outcomes

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## Abstract

**Background—**Previous studies in infant rats and case-control studies of human infants undergoing surgery have raised concerns about potential neurodevelopmental toxicities of general anesthesia. Spinal anesthesia is an alternative to general anesthesia for some infant surgeries. To test for potential toxicity, we developed a spinal anesthesia model in infant rats.

**Methods—**Rats of postnatal ages 7, 14, and 21 days were assigned to: no treatment; 1% isoflurane for either 1 h or 6 h, or lumbar spinal injection of saline or bupivacaine, at doses of 3.75 mg/kg (low dose) or 7.5 mg/kg (high dose). Subgroups of animals underwent neurobehavioral testing and blood gas analysis. Brain and lumbar spinal cord sections were examined for apoptosis using cleaved caspase-3 immunostaining. Lumbar spinal cord was examined histologically.

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Rats exposed to spinal or general anesthesia as infants underwent Rotarod testing of motor performance as adults. Data were analyzed using analysis of variance (ANOVA) using general linear models, Friedman Tests, and Mann–Whitney U tests, as appropriate.

**Results—**Bupivacaine 3.75 mg/kg was effective for spinal anesthesia in all age groups, and produced sensory and motor function recovered in 40 to 60 min. Blood gases were similar among groups. Brain and spinal cord apoptosis increased in rats receiving 6 h of 1% isoflurane, but not among the other treatments. All groups showed intact motor performance at adulthood.

**Conclusions—**Spinal anesthesia is technically feasible in infant rats, and appears benign in terms of neuroapoptotic and neuromotor sequelae.

The potential for harmful effects of general anesthesia on the developing brain has been described as pediatric anesthesiology's greatest current challenge.1 Anesthetic agents appear to have the greatest neurodegenerative impact when exposure occurs during the period of rapid synaptogenesis.2-15 Among species, peak synaptogenesis occurs at different rates and at different parts of the life cycle. This critical period of synaptogenesis in the rat lasts from roughly day of birth to day 10, peaking in cortex at day 7. In humans, this corresponds roughly to a period from the third trimester of gestation and the initial 3 yr of postnatal life. In rats and mice, brain growth is most rapid during the first 2 weeks of postnatal life. The brain's maturational state in postnatal day 7 mice is comparable to 7-day-old rats and corresponds to premature human neonates.16,17

Studies in infant rat models have shown that exposure to a number of classes of sedative-hypnotic medications, including benzodiazepines, n-methyl-d-aspartate receptor antagonists, and inhalation anesthetics produce striking degrees of apoptotic neurodegeneration, particularly at 7 days postnatal age (P7) but not at postnatal days 21 (P21). Similar concerns have been raised by studies in other species, including infant mice young primates and in brain slice preparations *in vitro*.2-15 Conversely, other investigators, including one of us (S.S.), have found increased brain apoptosis in sections of parietal and temporal cortex and thalamus in P7 rats with prolonged (6-h) ketamine exposure, but not with brief (1-h) exposure.4,18 During programmed cell death, also termed apoptosis, a cascade of autodigestive enzymes known as caspases are activated. Cleavage of the final member of this cascade, caspase-3, will leads to activation of endonucleases, resulting in apoptotic cell death.19

Case-control studies have shown that infants undergoing surgery under general anesthesia, especially with more than one procedure20 may have a higher frequency of neurodevelopmental difficulties than infants who did not require anesthesia in infancy.20 Conversely, a recent twin study did not find evidence for harmful sequelae of anesthesia in infancy.21 Case-control studies do not fully answer the question of whether the anesthesia *per se* is associated with increased risk, due to the confounding effect of surgery, postsurgical stress, and other comorbidities that may be more common among infants requiring surgery.

To address these concerns, a large international randomized controlled trial is in progress examining neurodevelopmental outcomes at 2 and 5 years of age in a group of infants undergoing inguinal hernia repairs under spinal or general anesthesia.22 The rationale for this trial is based in part on the assumption that spinal anesthesia would not be expected to produce harmful effects on neurodevelopment. Nevertheless, this assumption has not previously been examined in infant animal surrogate models.

The present study describes a technique for spinal anesthesia in the young rat. Our general hypothesis is that spinal anesthesia is neurologically benign in young rats. In particular, we

hypothesized that (1): a spinal bupivacaine dose could be identified that produced signs of lower body blockade without signs of high spinal blockade, severe physiologic instability, or high mortality, (2) these doses would not increase apoptotic neurodegeneration in the brain or spinal cord (as assayed by cleaved caspase-3 staining), as seen with prolonged exposure to a volatile anesthetic, (3) spinal anesthesia would not produce long-term deficits in motor performance in those rats when tested as adults, and (4) histologic effects of spinal anesthesia on lumbar spinal cord would appear benign.

## Materials and Methods

### Animals

With the approval of the Investigational Review Board and adherence to the Guide for the Care and Use of Laboratory Animals,23 all experiments utilized Sprague-Dawley postnatal day 7 (P7), 14, (P14) and 21 (P21) male and female rat pups (Charles River Laboratories, Wilmington, MA). They were housed in a room on a 12-h light/dark cycle with a free access to water. Rat pups were kept in cages with their littermates and mothers until weaning at postnatal age 21 and were housed with their littermates thereafter.

The experiments were conducted in a temperature-controlled acrylic container maintained at 36.7°C. In order to minimize handling of the pups, direct core temperature measurements were not obtained. However, similar conditions resulted in core body temperatures between 36.5 and 37.5°C.11

The rat pups were handled repeatedly by investigators to diminish effects resulting from stress-induced analgesia. The P7 and P14 rats were separated from their mothers during spinal injection and behavioral testing but were promptly returned for warmth and breast-feeding thereafter. The P21 rats were allowed free access to water and food at all times except during the spinal injection and behavioral testing. Rat pups for each age group were randomly assigned to spinal and general anesthesia as described in the sections to follow on “Intrathecal Injections of Bupivacaine” and “General Anesthesia Exposure”

### Intrathecal Injections of Bupivacaine

The P7, P14, and P21 rats underwent baseline measurement of hind paw thermal withdrawal latencies (see Behavioral Measurements section below) immediately before spinal injection. The content of the spinal solutions were blinded to the investigator who performed the intrathecal injections (BY). With the animals in a prone position, the spinal solutions were injected intrathecally at the L4–L5 or L5–L6 level using a 100 μl syringe (26S gauge needle, model 801 RN; Hamilton Company, Bonaduz, Switzerland). Intrathecal placement of the needle tip was confirmed by observation of a tail flick.

Preliminary experiments indicated that awake P7 rats could be held un-anesthetized sufficiently still to perform these injections with high success rates, but that sufficient immobility was more difficult to achieve in awake P14 and P21 rats. For this reason, experiments in P14 and P21 rats were all performed after brief (less than 2 min) inhalation of isoflurane 2–4% (Baxter Healthcare Corporation, Deerfield, IL) vaporized in oxygen. The experiments in P7 rats were performed with separate groups of rats either awake or under brief isoflurane-anesthetized conditions, respectively. Doses were varied by maintaining a constant concentration of bupivacaine 7.5 mg/ml commercial solution (Hospira, Inc., Lake Forest, IL) and injecting varying volumes scaled to the rat pup’s body weight. Injected doses were 3.75 mg/kg as a low dose and 7.5 mg/kg as a high dose in all rats. Following spinal injections, animals were placed in a warm flat cage and thermal latencies were measured every ten minutes until full recovery and for a minimum of 40 min after injection. All rats receiving bupivacaine had successful blocks, as determined by signs of thermal blockade.

### General Anesthesia Exposure

Groups of rats were exposed to either 1 h or 6 h of 1% inspired concentration of isoflurane in oxygen in a temperature-controlled chamber. Isoflurane was delivered using calibrated Matrx vaporizer (Matrx Medical Inc, Orchard Park, NY). Isoflurane and oxygen concentration were monitored by Capnomac Ultima Anesthesia Monitor (Datex-Ohmeda, Instrumentarium Corp., Helsinki, Finland) and animals were visually inspected for respiratory effort and skin color. While many previous publications on prolonged general anesthetic exposure in infant rats have not provided glucose supplementation during the anesthetic, there remains a concern that the neurologic effects of prolonged general anesthesia could be exacerbated by lack of substrate availability.7 In order to account for this possibility, separate groups of animals at each of the three ages receiving 6 h isoflurane exposures were treated with and without dextrose supplementation, respectively. Supplementation involved intraperitoneal injections of 5% dextrose, 0.05 ml, given hourly beginning after 1 h of anesthetic exposure.*

### Methylene Blue Injections

Pilot experiments were performed to evaluate the spread of injectates in the spinal subarachnoid space. Using the same technique and age-specific injection volumes described above, rats received spinal injections of methylene blue 1 mg/ml solution. The extent of spread of the methylene blue was examined upon animal necropsy 10 min after intrathecal injections.

### Behavioral Assessments for Sensory and Motor Blockade

Blockade of thermal nociception was assessed using a modified hot plate test, similar to what we have used previously in studies of peripheral nerve blockade.24,25 Hind paws were exposed in sequence (left then right) to a hot plate (model 39D hot plate analgesia meter; IITC Inc., Woodland Hills, CA) at 52°C for P7 and P14 pups and 56°C for P21 adolescents. The time (thermal withdrawal latency) until the rats lifted their paws was measured with a stopwatch. After 12 s, the tested paw was removed by the experimenter to avoid injury to the animal or the development of hyperalgesia. This test was repeated three times (with a 10-s pause between tests) for each rat at every time point. Thermal withdrawal latencies were measured every 10 min for at least 40 min after the intrathecal injection and until full recovery was observed.

Blockade of mechanical nociception was assessed by hind paw withdrawal to von Frey filaments. A series of filaments of increasing bending force was applied, and the force in grams of the filament that first evoked a withdrawal response was recorded. Mechanical withdrawal thresholds were recorded at baseline and every 10 min for at least for 40 min after the intrathecal injection and until full recovery was observed. Of note, for both the thermal and mechanical withdrawal tests, animals were observed for the possibility of exhibiting motor blockade without sensory blockade, *i.e.*, by absence of lower limb movement accompanied by vocalization or signs of upper body escape responses. This was not observed for any animal.

Motor performance of the lower extremities was assessed by a qualitative score. For each leg, if there was no spontaneous or evoked movement, the contribution to the score was zero. If there was partial movement, the contribution was one, and if there was normal movement, the contribution to the score was two. Thus, in summing the values for both legs, the score could range from zero (complete blockade) to four (normal).

*Explanation is provided at http://vetmed.duhs.duke.edu/guidelines_for_postoperative_supportive_care_in_rodents.htm (11/30/2010)

### Motor behavior on Postnatal day 30

Motor impairment was assessed on the P30 rats that had undergone spinal bupivacaine or saline injections or 1 h or 6 h of 1% isoflurane general anesthesia interventions at postnatal day 7. These rats were introduced to a Dual species Economex Rotarod (Columbus Instruments, Columbus, OH) using a spindle rotating at 10 rotations per minute.11 Each rat was tested three times with 10 min intervals between each assessment. The maximal latency for each trial was 300 s before removal from the spindle. The average of the three assessments was used for data analysis. The investigator was blinded to the treatment groups.

### Euthanasia and Perfusion

The animals were euthanized with intraperitoneal injection of sodium pentobarbital 100 mg/kg (Hospira, Inc.) and perfused transcardially with saline followed by 4% paraformaldehyde 6 h after the treatment periods ended. The spinal anesthesia groups (receiving bupivacaine or saline control), sham group, and 1-h isoflurane or 6-h isoflurane groups were perfused 6 h following initiation of the anesthetic intervention. After gentle dissection of the brain and spinal cord, the organs were stored overnight at 4°C in 4% paraformaldehyde and then transferred to a 30% sucrose solution at 4°C until sectioning, as detailed below. All samples were coded to allow for blinded histologic evaluation.

### Blood Gas Analysis

A cohort of P7, P14, and P21 rats treated with spinal saline, spinal anesthesia at high and low dose, 1 h or 6 h (with or without dextrose as detailed above in the section on General Anesthesia Exposure) of 1% isoflurane general anesthesia underwent cardiac puncture 10 min after either spinal injection or the end of general anesthesia for the blood gas analysis. For all groups receiving spinal injections, appropriate presence or absence of blockade was confirmed by neurobehavioral criteria as per above. Rats were terminally anesthetized by intraperitoneal injection of sodium pentobarbital 100 mg/kg, the heart was exposed, and 0.3 ml of blood was taken by right ventricular cardiac puncture, for immediate blood gas analysis using a blood gas analyzer (pHOx Plus L, Nova Biomedical, Mississauga, Ontario, Canada).

### Cleaved caspase-3 Immunohistochemistry

Fixed brains and spinal cords were placed in dry ice for 10–15 min and placed gently on a thin layer of Frozen Tissue Embedding Media (Fisher Diagnostic, Fairlawn, NJ) inside the cryostat box to create a block for sectioning. The brains and spinal cords were sectioned at 20 μm thickness using a cryostat (Leica CM3050S, Bannockburn, IL). Each slide contained 4 brain sections or 6 spinal cord sections. Coronal brain sections were obtained at a location that includes the lateral dorsal thalamus, ventrolateral division, and corresponds to an anteroposterior plane approximately 2 mm from bregma in the adult rat, similar to figure 26 in the Paxinos and Watson atlas.26 and lumbar sections of spinal cord were mounted onto Super Plus –coated glass slides (Fisher Scientific, Pittsburgh, PA). The slides were allowed to dry at room temperature for 24 h. Initially, the blocking solution (bovine-serum-albumin phosphate-buffered-saline Triton × 100 -Sodium Azide) was applied on the sections for half an hour at room temperature and then the blocking solution was removed from the sections to apply the primary rabbit anticleaved caspase-3 antiserum (1:2,500, Cell Signaling, Danvers, MA). Slides were then incubated overnight at room temperature. On the next day the sections were rinsed 3 times with phosphate-buffered saline, and then secondary antibody CY3 anti rabbit (1:100, Jackson Immunoresearch, West Grove, PA) was applied. Slides were incubated at room temperature for 2 h. After 2 h, secondary antibody was removed and the sections were washed three times in phosphate buffered saline and then

given a final wash with 0.0 5M phosphate-buffered. Finally, the slides were allowed to dry before they were mounted over 3 drops of 90% glycerol with a cover slip. Microscopic evaluation was done using a BX-60 light microscope (Olympus, Southall, United Kingdom) at 20× magnification. A Hamamatsu digital camera C10600 ORCA-$R^2$ (Photonic K.K., Hamamatsu City, Shizuoka, Japan) was used to take images of each section.

The investigator, blinded to the experimental cohort, counted the number of cleaved caspase-3 positive cells on one slide per animal either at the cortex in four brain sections or in six sections at the lumbar spinal cord. The number of cells counted reflected the sum of the cell counts per animal.

### Histopathology of the spinal injection site

Animals from each age group were used for histopathologic evaluation of lumbar spinal cord. For P7, 10 animals were used, with five of the animals were treated with intrathecal injection of bupivacaine 3.75 mg/kg and five were injected with the same volume of saline. For P14 and P21, four animals each received intrathecal bupivacaine 3.75 mg/kg or saline, respectively. Rats received spinal injections as detailed above, and they were allowed to recover in their cages. All rats receiving bupivacaine (*i.e.*, not saline controls) had successful blocks, as determined by signs of sensory and motor blockade per above. Seven days following injections, they were terminally anesthetized with Tribromoethanol (Avertin®, σ, St. Louis, MO) 2.5% 20 ml/kg, and underwent cardiac puncture for perfusion with cold phosphate buffered saline 30 ml, followed by 50 ml of cold fixative, containing 1.25% paraformaldehyde, 2.5% glutaraldehyde, 0.03% Picric Acid in 0.1M Cacodylate buffer. In brief, the histologic approach involved: fixation for 48 h, washing 2× in 0.1M cacodylate buffer, post fixation incubation for 48h in 1% OsO4 in 0.1M cacodylate buffer, washing - 2× 1h 0.1M cacodylate buffer, dehydrating in graded ethanol solutions: 50, 75, 95 100%(2×), suspension in Propylene Oxide: 1h - 2x, Propylene Oxide : araldite-ddsa (Epon) 1:1: overnight, embedding in epon, and then sectioning in 1 μm sections. Sections were mounted on slides, counterstained with Epoxy Tissue Stain (EMS, Hatfield, PA) and covered. Slides were coded and images have been taken at 10× and 60× magnifications with a Hamamatsu digital camera. The images were then examined by an expert in nerve structure and pathology (G.C.) who was blinded to the treatments.

For P14 and P21 rats, where there is sufficient maturation of tracts in the white matter of the lumbar spinal cord by the time of sacrifice 1 week after injections, a semiquantitative rank ordered scoring system was used, as per Estebe and Myers (2004).27 with the following 6 point Likert scale: zero was no pathology in any portion of the field, 1 was very few myelinated axons with any mild abnormality, 2 was slightly more myelinated axons with any abnormality than 1, but less than 10% abnormal, 3 referred to 10–20% abnormal myelinated axons, 4 referred to signs of moderate axonal degeneration, and 5 referred to clear signs of axonal degeneration. Since P7 rats, when sacrificed 1 week later at P14, have much less developed myelination and immature laminar organization of the spinal cord, a more qualitative interpretation was used by the examiner, who rated slides only as normal or not normal for age.

### Statistical Analysis

Thermal withdrawal latencies and von Frey mechanical withdrawal thresholds before and after bupivacaine or saline intrathecal injection was compared using a two-way mixed-model factorial analysis of variance (ANOVA) with age group (P7, P14, P21) and treatment (saline, bupivacaine 3.75 mg/kg, bupivacaine 7.5 mg/kg) as factors and F-tests were used to compare ages and treatments with a conservative *post hoc* Sidak-Bonferroni correction for multiple comparisons to protect against Type I errors. Blood gas parameters were compared

between 6 treatment groups (spinal saline, spinal bupivacaine 3.75 mg/kg, spinal bupivacaine 7.5 mg/kg, isoflurane 1% 1h, isoflurane 1% 6h, isoflurane 1% 6h + dextrose supplementation) using analysis of variance (ANOVA) with Dunnett's *post hoc* procedure to assess mean differences relative to the spinal saline (control) reference group. Hind leg motor response data are presented as median and interquartile range and compared between treatment groups for each age using the two-way nonparametric Friedman test using time as a repeated measures factor. Motor performance as measured by the Rotarod apparatus was compared between 4 groups (saline, bupivacaine 3.75 mg/kg, isoflurane 1% 1h, isoflurane 1% 6h) also by ANOVA with the F test used to ascertain differences with 95% confidence intervals constructed around the mean performance. Apoptosis as measured by caspase-3 activation using immunohistochemistry was compared between treatment groups in P7 rat pups by one-way ANOVA. Histologic injury scores were compared between spinal saline and spinal bupivacaine groups at ages P4 and P21 using Mann–Whitney U tests. Two-tailed values of $P < 0.05$ with correction as appropriate were considered statistically significant. Statistical analysis was performed with SPSS version 18.0 (SPSS Inc/IBM, Chicago, IL).

## Results

Among the rats used, the postnatal day 7 (P7) rat pups weighed an average and SD of 16.23 ± 1.9 g, the P14 rat pups weighed an average of 32.2 ± 2.4 g, and the P21 rats weighed an average of 48.8 ± 4.3 g.

### Technique, Pilot Experiments, and Initial Dose-Finding

Needle placement evoked a tail flick in all rat pups in one or two attempts. Spinal injections were performed only when a tail flick was observed. Pilot experiments using escalating doses of bupivacaine found that 10 mg/kg produced respiratory failure and death in 4 of 5 P7 rat pups. Preliminary experiments using bupivacaine 7.5 mg/kg dose injected over 30 s produced behavioral signs of high spinal block, including weakness and reduced response to pinch in the forepaws, labored respiration, and death in 4 out of 17 animals at ages P7 (23.5%), 5 out of 19 at P14 (26.3%), and 5 out of 20 at P21 (25%), respectively. Subsequent experiments showed lower death rates (less than 5%) with the 7.5 mg/kg dose when the dose was injected over 1 min and when the isoflurane was discontinued as soon as the tail flick was identified. Pilot experiments with bupivacaine 3.75 mg/kg showed preservation of motor and sensory function in the forepaws, and normal respiratory efforts. Additional animals received intrathecal injection of methylene blue 1 mg/ml in volumes age-matched to equal the 3.75 mg/kg bupivacaine dose (8 μl for P7, 16 μl for P14 and 25 μl for P21). Necropsy showed a subarachnoid spread of dye to thoracic levels.

In pilot experiments in P7 rats, needle placement either awake or under a 2-min isoflurane anesthetic (using an inhalation chamber followed by a nasal cone) gave equivalent technical success and time course of spinal anesthesia.

### Effect of Intrathecal Bupivacaine on Behavioral Measures of Sensory and Motor Blockade

**Withdrawal to a Thermal Stimulus—**Hind paw thermal withdrawal latencies were determined for P7 (fig. 1A), P14 (fig. 1B), and P21 (fig. 1C) rats in groups receiving spinal bupivacaine at high dose (7.5 mg/kg) or low dose (3.75 mg/kg), or saline controls. For all age groups, both the 3.75 mg/kg and 7.5 mg/kg bupivacaine doses produced dense thermal nocifensive blockade at the first measurement at 10 min following injection, and remained dense at 20 min. In P21 rats, thermal withdrawal latencies remained significantly greater than control values at 30 and 40 min for the high dose bupivacaine treatment, and at 30 min for the low dose bupivacaine treatment. In P14 rats, thermal withdrawal latencies remained significantly greater than control at 30 and 40 min for the high dose, but not low dose

bupivacaine treatment. In the P7 rats, thermal withdrawal latencies remained significantly above baseline at 30 min for both high dose and low dose groups. Rats receiving intrathecal saline showed no impairment of responses to thermal stimuli in any age group (fig. 1).

**Withdrawal to a Mechanical Stimulus—**Withdrawal responses to Von Frey filaments applied to the hindpaws are shown in figure 2 for P7 (fig. 2A), P14 (fig. 2B), and P21 (fig. 2C) rats in groups receiving spinal bupivacaine at high dose (7.5 mg/kg) or low dose (3.75 mg/kg), or saline controls.

**Motor Block Scores—**Motor block scores are shown in table 1. For all age groups, no animal receiving spinal saline showed any signs of motor impairment. Bupivacaine at both low dose (3.75 mg/kg) and high dose (7.5 mg/kg) produced dense motor block in all animals that recovered completely by 40 min in all low dose animals for all age groups and by 50 min in all high dose animals for all age groups. Nonparametric analysis using the Friedman test indicated longer motor block for each bupivacaine treatment group compared to saline for at least the first 20 min, with the high dose bupivacaine of 7.5 mg/kg lasting longer than 3.75 mg/kg (table 1).

**Response to Pinch—**Animals receiving saline injections all responded to pinch with forceps on the skin of the back with a startle, escape behaviors and turning of the head and movement of the forepaws. All animals receiving spinal injections with bupivacaine 3.75 mg/kg showed no behavioral response to pinch over the skin of the back at lumbar and lower thoracic levels, but they had identical brisk withdrawal behaviors to pinch at upper thoracic levels and on the forepaws. Animals receiving spinal injections with bupivacaine 7.5 mg/kg showed no behavioral response to pinch over the skin of the back at lumbar and lower thoracic levels. As noted above, in pilot experiments, some animals died with this dose of bupivacaine, and others appeared sedated for 1–2 min, and then recovered. While some sedated animals had sluggish responses to pinch at upper thoracic levels and on the forepaws for 1–2 min, all animals that survived showed brisk withdrawal behaviors to pinch at upper thoracic levels and on the forepaws by the 10 min testing point.

**Response to Surgical Incision—**Separate groups of P7, P14, and P21 animals underwent spinal anesthesia with bupivacaine 3.75 mg/kg, and then underwent full thickness incision of the hindpaws. No animal responded to the incision with any vocalization, flinch, head turning, upper body movement or escape response. Subsequent pinch of the area of the hind paw incision also failed to induce any behavioral response.

### Blood Gas Analyses

Mixed venous (right ventricular) blood samples were analyzed for blood gases, glucose and lactate for groups of P7 rats that underwent spinal injections of saline, bupivacaine 3.75 or 7.5 mg/kg or 1 and 6 h of 1% isoflurane general anesthesia are shown in table 2. Two 6 h isoflurane anesthesia groups were examined: one with and one without intraperitoneal supplementation of dextrose and fluid. General linear model ANOVA showed no overall effect of treatment on values of any parameter, except that the supplemental dextrose group had increased blood glucose concentration and slightly reduced bicarbonate concentrations compared to the other groups (table 2). Blood lactate concentrations were not statistically different among groups. In particular, they were not increased in the groups received 1% isoflurane for 6 h, with or without dextrose supplementation (table 2).

### Motor Performance in Adult Rats with Postnatal Anesthetic Exposures

Groups of rats at 30 days of age who had undergone exposure at P7 to spinal bupivacaine, spinal saline, isoflurane for 1 h, or isoflurane for 6 h, respectively, were tested for motor

performance using the Rota Rod apparatus. No group differences were found for this test. The times that rats remained on the Rota Rod (mean + SD) for the saline, spinal bupivacaine. 3.75 mg/kg, 1 h Isoflurane and 6 h Isoflurane treated groups were 272 ± 30, 255 ± 25, 278 ± 27 and 265 ± 27 s, respectively (table 3).

### Additional Behavioral Observations

No animals were observed to have swelling at the injection sites, or signs of distress, hyperalgesia, or self-mutilation. Furthermore, none of the animals had any evident residual weakness or impairment of gait when reassessed at 24 h following spinal injections.

### Cleaved Caspase-3 Staining for Apoptotic Neurodegeneration in the Brain and Spinal Cord

Blinded counts of cleaved caspase-3 staining cells showed no increase in apoptosis in brain or spinal cord in rats receiving spinal anesthesia as compared to controls or 1 h exposure to 1% isoflurane, but were significantly less than those with 6 h exposure to 1% isoflurane (fig. 3)

Our analysis revealed an overall effect of condition on average number of apoptotic cells in brain ($F = 34.98$, P less than 0.001, $df = 44$) and spinal cords ($F = 8.48$, P less than 0.001, $df = 35$). *Post hoc* Dunnett's tests found that only the group with exposure to 1% isoflurane for 6 h showed a significant increase in the number of apoptotic cells in both brains and spinal cords compared to controls (fig. 3)

### Histologic Appearance

To test the possibility that intrathecal bupivacaine injection has effects on spinal cord structure, we examined tissues (fig. 4) from animals injected with saline (fig. 4A, 4C, 4E) or Bupivacaine (3.75 mg/kg) (fig. 4B, 4D, 4F) seven-days after treatment. Examination of plastic embedded spinal cord sections of animals injected at P7 (fig. 4 A & B), P14 (Fig. C & D) and P21 (fig. 4 E & F) showed no differences between saline and bupivacaine injected animals at all ages. We found no alterations in the appearance of myelinated fibers, cell bodies or perineural tissues. No signs of histopathology were noted at any age group. For the P14 animals, histologic injury scores in white matter, as outlined in Methods, were a median of 2 (range 1–5) in the spinal saline group and a median of 1 (range 0–2) in the spinal bupivacaine 3.75 mg/kg group. The single animal rated as "5" in the saline group was felt by the examiner to have had probable artifact due to processing or tissue trauma, rather than any sign of demyelination or neuronal injury. No other animal had a score above 2. For the P21 animals, median scores were 0 (range 0–0) in the saline group and 0 (range 0–2) in the bupivacaine group, respectively. For the P7 animals, all slides in both the spinal saline and spinal bupivacaine groups were graded as normal for age.

## Discussion

This study developed a model for spinal anesthesia with bupivacaine in infant rats, and identified a dose that produced anesthesia with apparent safety and tolerability. A comparison of the treatment groups demonstrated that there were no significant differences in the appearance of cleaved caspase-3 cells in sections of brain and lumbar spinal cord between the control, bupivacaine, and 1 h isoflurane groups. However, the 6 h isoflurane group showed significantly higher numbers of cleaved caspase-3 cells than the other treatment groups. The effect of prolonged isoflurane anesthesia on apoptosis could not solely be ascribed to lack of substrate from prolonged fasting, since rats who did not receive supplemental dextrose during the prolonged anesthesia did not show either hypoglycemia or lactic acidosis, and since dextrose supplementation even to the point of mild hyperglycemia did not prevent apoptosis. These observations are consistent with previous studies that

demonstrated isoflurane –induced neuroapoptosis without detectable changing in physiologic parameters as noted above.7,8

Despite the significant increase in cleaved caspase-3 cells in the 6 h isoflurane group, no motor deficits were detected between the groups in Rotorod testing at 30 days of age. This is consistent with the finding that prolonged isoflurane exposure in neonatal mice led to increased apoptosis, but no decrements in spontaneous locomotion in adulthood.28 As the current investigation only tested motor performance, we cannot exclude effects on other more subtle neurologic outcomes based on these data.

Cleaved caspase-3 serves as a marker for apoptosis in the detection of anesthetic-induced developmental neurotoxicity. Exposure to 0.75% isoflurane and 75% nitrous oxide for 6 h resulted in increased cleaved caspase-3 cells in the spinal cord of P7 rat pups.11 Despite the increase in neuroapoptosis after exposure to isoflurane and nitrous oxide, no functional deficits were detected in the tail flick latency or Rotorod tests. Our findings confirm an increase in apoptosis in the brain and spinal cord of neonatal rats exposed to 1% isoflurane for 6 h. However, cleaved caspase-3 counts in neonatal rats receiving an intrathecal bupivacaine or 1% isoflurane for 1 h were not different from their control cohorts. A previous report in mice notes that a 1 h exposure to 2% isoflurane, double the concentration used in the current study, resulted in increased cleaved caspase-3 expression.7 The discrepancy between our findings might be explained by the difference in concentration of isoflurane and the species utilized in these experiments.

The neurotoxic effect of local anesthetics on the spinal cord has been studied extensively in adult rats.29 Bupivacaine appears not to be toxic to the spinal cord in older rats, although general anesthesia has been shown to induce apoptotic neurodegeneration in the neonatal rat spinal cord.11 Our observations in neonatal rats show that intrathecal bupivacaine had a similar morphological and apoptotic profile as the 1 h isoflurane and control groups. Furthermore, the motor function tested at 30 days showed no differences between the experimental groups.

The specter of developmental neurotoxicity of anesthetic agents in laboratory animals has incited the pediatric anesthesia community to examine their clinical practices. Several investigators have demonstrated that drugs commonly used for general anesthesia and sedation produces accelerated neurodegeneration and neurobehavioral deficits. Regional anesthetic techniques are routinely utilized as alternatives to general anesthesia in appropriate surgical procedures in pediatric patients. However, the effect of spinal anesthesia on the developing central nervous system has not been previously investigated. Infant animal studies have the theoretical potential to detect age-specific toxicities and thereby prevent harm to human infants. In the current study, we show that spinal anesthesia with bupivacaine is technically feasible in the infant rat, and that a dose of 3.75 mg/kg appears to produce thoracic-level sensory blockade, without evident signs of morbidity or distress. Rats that had undergone spinal or general anesthesia as infants showed no motor impairments as young adults on day 30 of life. Cleaved caspase-3 staining showed no increased frequency of neuronal cell death in spinal bupivacaine-treated animals, unlike those animals treated with isoflurane for 6 h. These findings demonstrate that spinal anesthesia does not produce neuroapoptosis in either brain or spinal cord; suggesting that it may be a feasible alternative to general anesthesia during the perinatal period.

The infant rat has been an important model for the study of the ontogeny of pain mechanisms30,31 and for the study of age-related differences in actions of anesthetic and analgesic drugs.32-35 Studies in infant rats and rhesus monkeys showing age-specific neurodegeneration following exposures to general anesthetics and sedative-hypnotics have

raised serious concerns about how this might apply to human infants undergoing surgery.36,37 Retrospective reports demonstrate a link between early and repeated exposure to general anesthetics to subsequent learning and behavioral deficits. As noted in the Introduction, some case-control human studies have reinforced these concerns. However, many factors complicate any attempt to generalize the results of these infant animal studies to infant humans. First, the experimental conditions under which data are collected from rodents differ from the conditions experienced by neonates undergoing surgery and general anesthesia.36,37 Second, there are important species differences in the time course of neurologic development and in the critical periods for neurogenesis and synapse formation. An ongoing prospective randomized controlled trial comparing spinal and general anesthesia in infants may further clarify the short-term and longer-term risks of both of these anesthetic techniques.22

## Conclusions

An infant rat model was developed to study immediate and longer term behavioral responses and potential brain neuronal loss following spinal anesthesia. Spinal bupivacaine, 3.75 mg/kg, produced hindlimb surgical anesthesia and did not induce apoptosis. As in previous studies, short-term exposure to subminimal alveolar concentrations of isoflurane appeared benign, while prolonged isoflurane exposure induced apoptosis. Within the limitations imposed by extrapolation from rats to humans, these experiments support the expectation that spinal anesthesia in infants should have benign neurodevelopmental effects.

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What we already know about this topic

- General anesthesia during infancy is associated in animals with enhanced apoptosis of neurons in brain, but spinal anesthesia has not been tested

What this article tells us that is new

- In infant rats aged 7 days, 6 h exposure to isoflurane induced brain apoptosis whereas spinal bupivacaine did not have this effect in either the brain or spinal cord

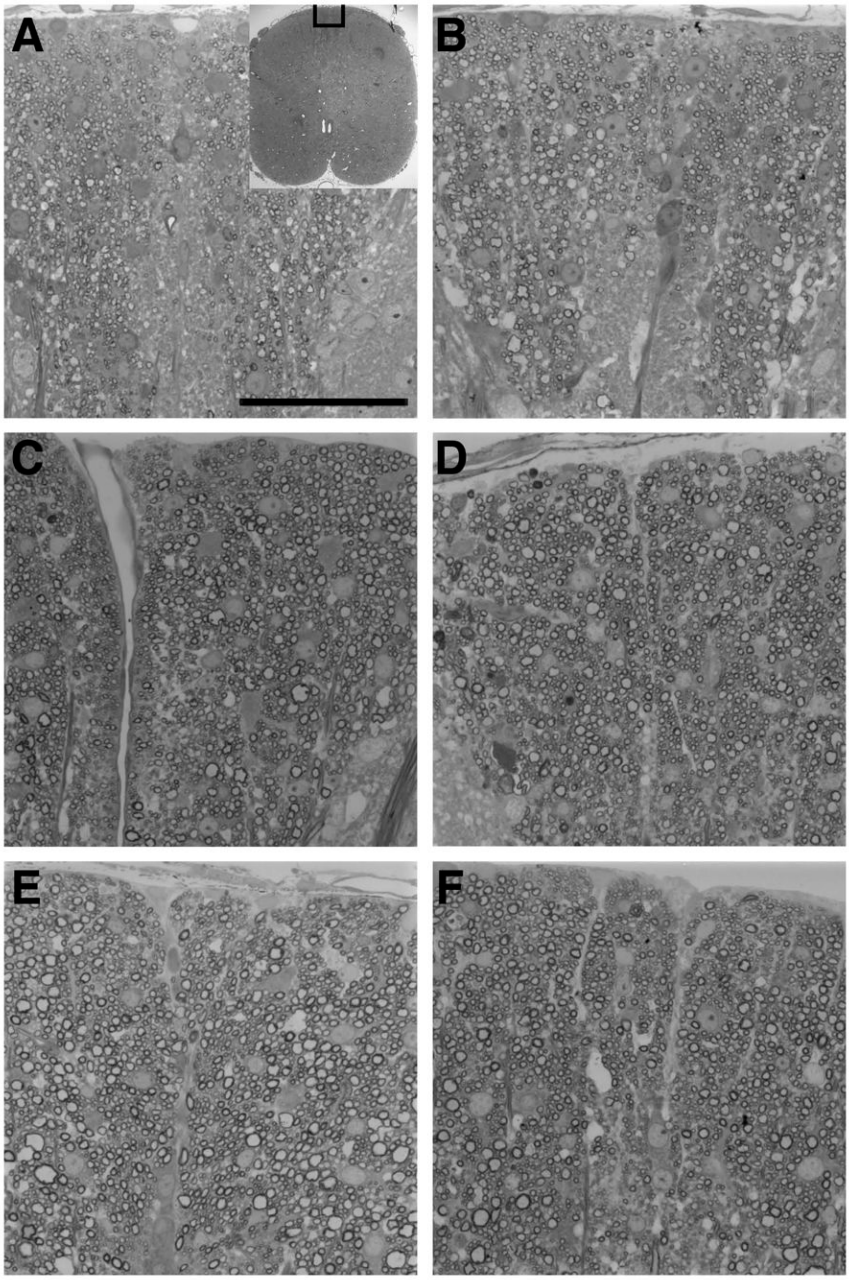


**Figure 1. Intensity and Duration of Thermal Nocifensive Blockade Using Spinal Anesthesia**

Lower extremity blockade, assessed by withdrawal latency to a modified hotplate test in rats at baseline (BL) and at times shown post-block, for P (post-natal day) ages: P7 (A), P14 (B), and P21(C), using low dose bupivacaine (3.75 mg/kg), high dose (7.5 mg/kg), or saline. Sample sizes of P7/P14/P21: n=9/8/9 (total n=26) for low dose bupivacaine, n=8/8/7 (total n=23) for high dose bupivacaine,and n=9/9/9 (total n=27) for spinal saline. * $P < 0.05$ compared to spinal saline. † $P < 0.05$ compared to low dose bupivacaine.

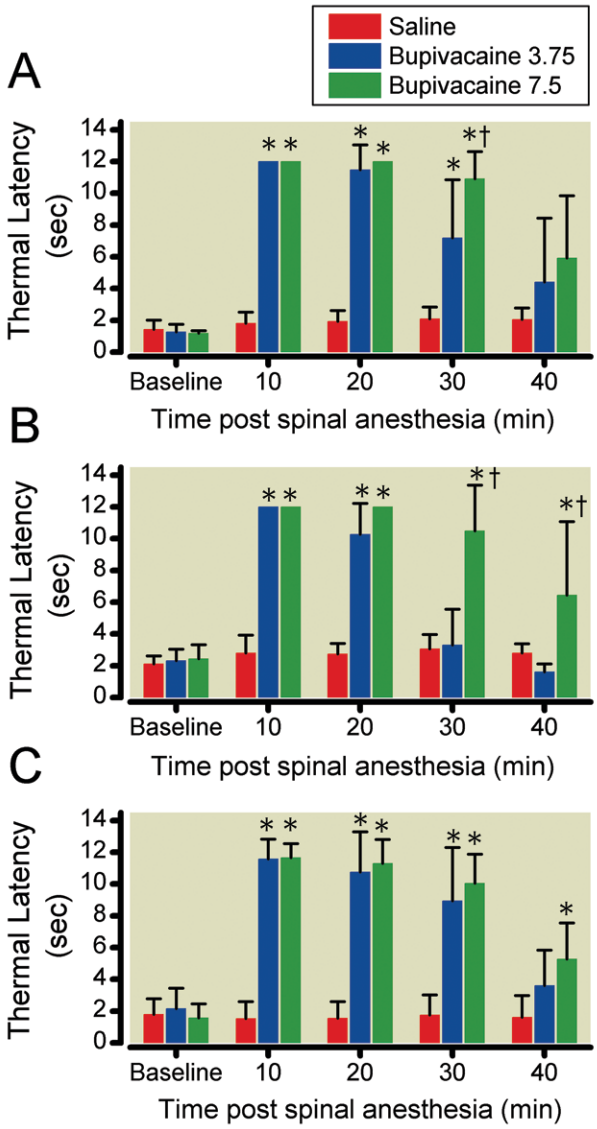


**Figure 2. Intensity and Duration of Mechanical Nocifensive Blockade Using Spinal Anesthesia**

Withdrawal thresholds (g) to von Frey filaments are shown for P (post-natal day) P7 rats (A), P14 rats (B), and P21 rats (C), respectively using low dose bupivacaine (3.75 mg/kg), high dose (7.5 mg/kg), or spinal saline. Sample sizes of P7/P14/P21: n=7/7/7 (total n = 21) for low dose bupivacaine, n=6/7/6 (total n=19) for high dose bupivacaine, and n=6/6/6 (total n=18) for spinal saline. * $P < 0.05$ compared to spinal saline. † $P < 0.05$ compared to low dose bupivacaine.

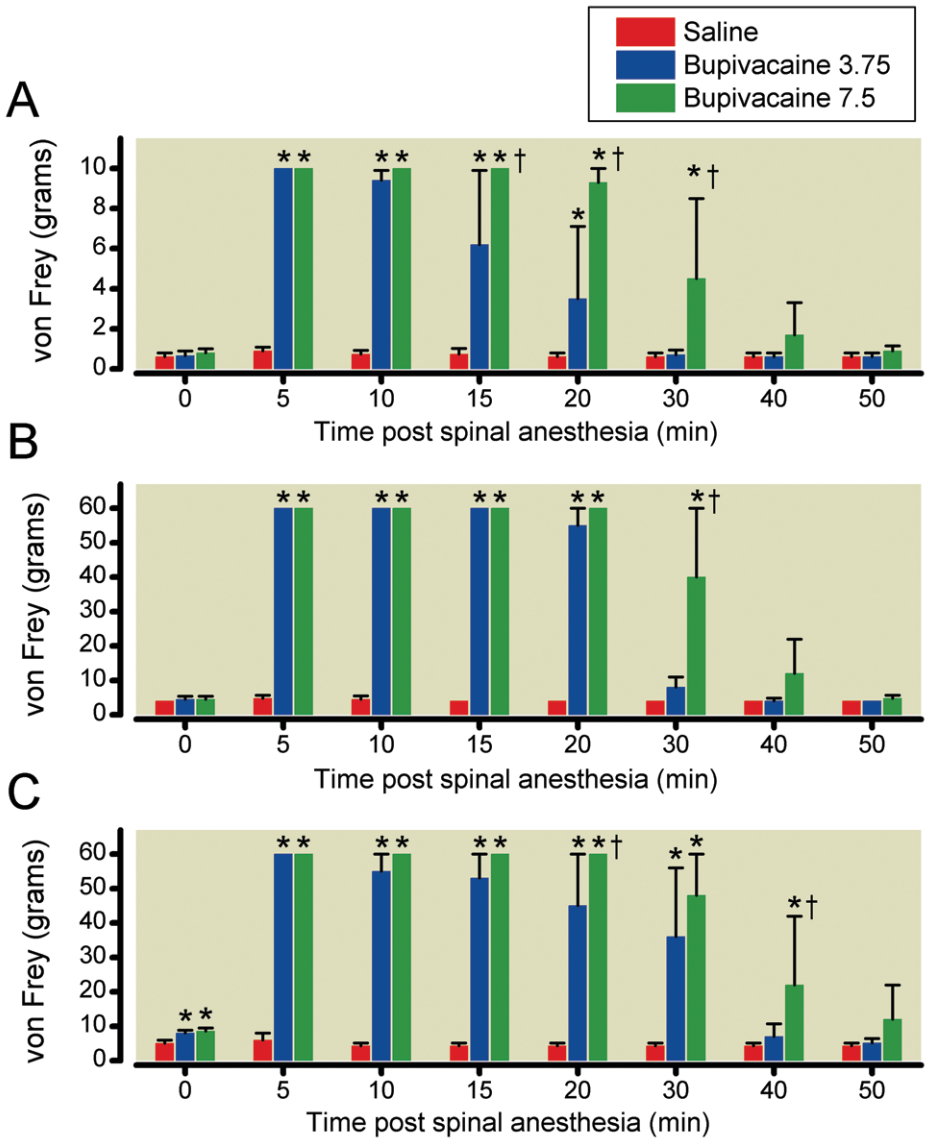


**Figure 3.**

Caspase-3 activation in the brain and spinal cord of P (post-natal day) P7 rat pups. Representative photomicrographs illustrate examples of cleaved caspase-3 immunocytochemical labeling in the somatosensory region of the cortex and spinal cord. Minimal labeling was found in brain (A,B) and spinal cord (E,F) sections from groups treated with spinal normal saline (A,E), spinal bupivacaine (B, F), or isoflurane 1 hour (not shown), while markedly increased labeled was seen in both brain (C) and spinal cord (G) in rats exposed to 6 hours of 1% isoflurane. Summary data on cleaved caspase-3 positive cells are shown for brain (D) and spinal cord (H). White arrows in panels (C) and (D) highlight the presence of cleaved-caspase-3 positive cells in the cortex and spinal cord, respectively. Sample size for all spinal cord labeling groups was 7 animals with the exception of isoflurane 1 hour (n=6) (total n=41). Sample sizes for brain labeling varied by group: n=12 for low dose spinal bupivacaine (performed awake), n=10 for spinal normal saline, n=6 for isoflurane 1 h, n=7 for isoflurane 6 hours, n=8 for no treatment, n=7 for low dose spinal bupivacaine performed under brief (<2 minutes) isoflurane general anesthesia (total n=50).

Data are presented as mean ± standard deviation, * $P < 0.05$ compared to other cohorts. Scale bar=100 μm.

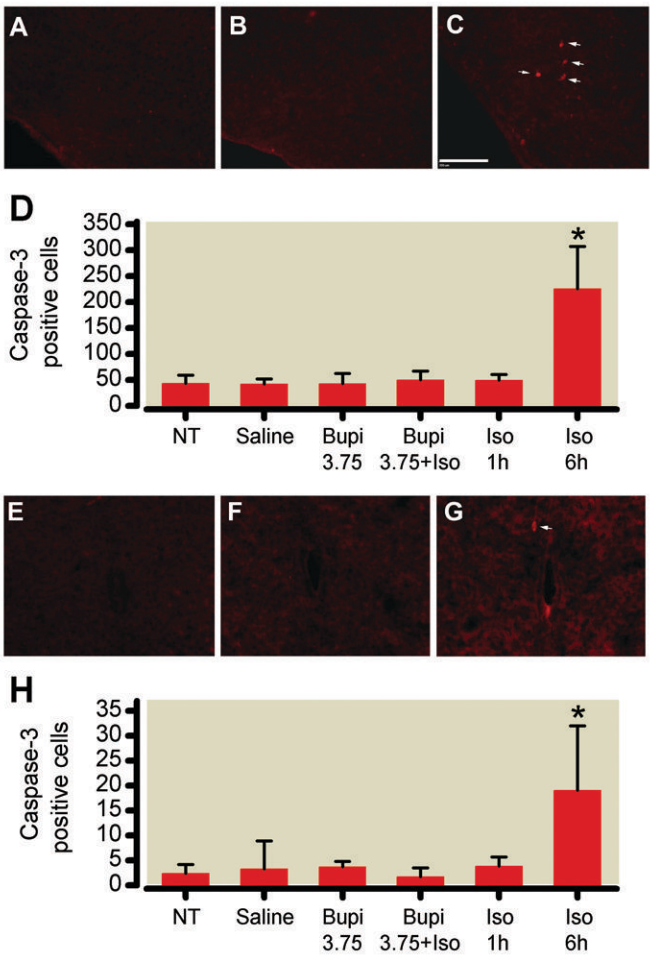


**Figure 4. Histological examination of the lumbar spinal cord following intrathecal administration of saline or bupivacaine**

Animals were injected with saline (A, C, E) or bupivacaine (3.75 mg/kg) (B, D, F) and seven days later their lumbar spinal cords were embedded in plastic, sectioned and analyzed for histopathology in a blinded fashion. n=5 for ages P (post-natal day) P7 across both treatment groups and n=4 for ages P14 and P21 across both treatment groups (total n=26). As detailed in the Results, no differences were observed between the treatments for any age group: P7 (A & B), P14 (C & D), and P21 (E & F). The insert in Panel A shows a lower magnification image of the spinal cord with a box depicting the area in which the high resolution analysis was performed. Scale bar 150 μm. * Bupi = bupivacaine. * Iso = Isoflurane. * NT = No treatment.

## Table 1

Comparison of Hind Leg Motor Response Between Spinal Saline and Bupivacaine Groups Stratified by Age

| Time (minutes) | Saline | Bupivacaine 3.75 mg/kg | Bupivacaine 7.5 mg/kg |
|---|---|---|---|
| P7 pups | (n = 6) | (n = 7) | (n = 6) |
| 0 | 4 (4-4) | 0 (0-0) * | 0 (0-0) * |
| 5 | 4 (4-4) | 0 (0-0) * | 0 (0-0) * |
| 10 | 4 (4-4) | 0 (0-0) * | 0 (0-0) * |
| 15 | 4 (4-4) | 1 (0-2) * | 0 (0-0) *† |
| 20 | 4 (4-4) | 2 (1-4) * | 0 (0-0) *† |
| 30 | 4 (4-4) | 4 (4-4) | 2 (0-3) *† |
| 40 | 4 (4-4) | 4 (4-4) | 4 (3-4) |
| 50 | 4 (4-4) | 4 (4-4) | 4 (4-4) |
| P14 pups | (n = 6) | (n = 7) | (n = 7) |
| 0 | 4 (4-4) | 0 (0-0) * | 0 (0-0) * |
| 5 | 4 (4-4) | 0 (0-0) * | 0 (0-0) * |
| 10 | 4 (4-4) | 0 (0-0) * | 0 (0-0) * |
| 15 | 4 (4-4) | 0 (0-0) * | 0 (0-0) * |
| 20 | 4 (4-4) | 0 (0-1) * | 0 (0-0) * |
| 30 | 4 (4-4) | 4 (3-4) | 1 (0-2) *† |
| 40 | 4 (4-4) | 4 (4-4) | 4 (3-4) |
| 50 | 4 (4-4) | 4 (4-4) | 4 (4-4) |
| P21 pups | (n = 6) | (n = 7) | (n = 6) |
| 0 | 4 (4-4) | 0 (0-0) * | 0 (0-0) * |
| 5 | 4 (4-4) | 0 (0-0) * | 0 (0-0) * |
| 10 | 4 (4-4) | 1 (0-1) * | 0 (0-0) * |
| 15 | 4 (4-4) | 1 (0-1) * | 0 (0-0) * |
| 20 | 4 (4-4) | 1 (0-2) * | 0 (0-0) * |
| 30 | 4 (4-4) | 2 (1-3) * | 1 (0-2) *† |
| 40 | 4 (4-4) | 4 (4-4) | 3 (2-4) *† |
| 50 | 4 (4-4) | 4 (4-4) | 4 (4-4) |

Data are median (interquartile range). Groups were compared by two-way nonparametric Friedman test. Scoring was 0-4 points, based on the sum of ratings for right and left sides. For each hind leg, 0 = no movement, 1 = partial impairment, and 2 = full movement. Sample sizes are given above (total n = 58). P = post-natal day.

* Statistically significant compared to saline ($P < 0.001$).

† Statistically significant compared to low dose bupivacaine ($P < 0.001$).

**Table 2**

Comparison of Blood Gas Parameters for the Treatment Groups

| Variable | Saline (n = 6) | Bupi 3.75 mg/kg (n = 6) | Bupi 7.5 mg/kg (n = 6) | Iso 1h (n = 6) | Iso 6h (n = 5) | Iso 6h + dex (n = 6) | ANOVA (F-test)* | *P* value |
|---|---|---|---|---|---|---|---|---|
| pH | 7.29 ± 0.05 | 7.34 ± 0.11 | 7.34 ± 0.07 | 7.29 ± 0.07 | 7.28 ± 0.06 | 7.26 ± 0.07 | 1.02 | 0.42 |
| $pO_2$ (mmHg) | 25.8 ± 5.1 | 32.3 ± 13.3 | 28.5 ± 9.6 | 20.2 ± 4.9 | 24.1 ± 3.5 | 27.8 ± 5.3 | 1.65 | 0.18 |
| $pCO_2$ (mmHg) | 75.7 ± 9.7 | 66.6 ± 19.3 | 63.2 ± 15.6 | 82.9 ± 5.6 | 75.7 ± 12.7 | 69.9 ±12.1 | 1.74 | 0.16 |
| $HCO_3$ (μmol/L) | 37.1 ± 2.5 | 35.3 ± 4.2 | 34.7 ± 4.3 | 40.6 ± 5.7 | 35.1 ± 2.49 | 31.6 ± 2.5 | 3.54 | 0.02 |
| Glucose (mg/dL) | 153 ± 19 | 158 ± 26 | 168 ± 16 | 159 ± 24 | 160 ± 22 | 211 ± 30 | 5.16 | <0.01† |
| Lactate (μmol/L) | 3.85 ± 1.16 | 4.4 ± 1.3 | 3.4 ± 1.4 | 5.07 ± 1.2 | 4.8 ± 1.5 | 5.0 ± 1.8 | 1.16 | 0.36 |

Data are mean ± Standard Deviation. ANOVA = analysis of variance. Bupi = bupivacaine. Dex = dextrose. $HCO_3$ = bicarbonate. Iso = isoflurane. $pCO_2$ = arterial partial pressure of carbon dioxide. $pO_2$ = arterial partial pressure of oxygen.

*F-test is based on 5 and 29 degrees of freedom for all variables, except lactate (5 and 26 degrees of freedom).

†Only significant difference versus saline is Iso 6h + dex group (ANOVA with *post-hoc* Dunnett tests). No significant group differences were detected for any of the other variables compared to saline.

## Table 3

Rotarod Performance for the Different Treatments*

| Treatment Group | Number | Mean ± SD, sec | 95% Confidence Interval |
|---|---|---|---|
| Saline | 4 | 272 ± 30 | 207 – 336 |
| Bupivacaine 3.75 mg/kg | 6 | 255 ± 25 | 202 – 307 |
| Isoflurane 1 hour | 5 | 278 ± 27 | 221 – 336 |
| Isoflurane 6 hour | 5 | 265 ± 27 | 207 – 323 |

SD = standard deviation.

*Analysis of Variance indicated no significant differences between the groups (F = 0.15, $P = 0.93$).

Rotarod motor performance testing was performed at P30 in rats receiving treatments on P7. There were no group differences in this performance measure. P = post-natal day.