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SCIENTIFIC ARTICLE

Effect of equipotent doses of bupivacaine and ropivacaine in high-fat diet fed neonatal rodent model

Ying-Dong Lian[a], **Zong-Xiang Chen**[a], **Kang-Ru Zhu**[b], **Shu-Yin Sun**[a], **Li-Ping Zhu**[b,*]

[a] *Jining No. 1 People's Hospital, Department of Emergency, Jining, China*
[b] *Jining No. 1 People's Hospital, Department of Pediatrics, Jining, China*

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KEYWORDS
Bupivacaine;
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Abstract
Objectives: The increase in the prevalence of obesity presents a significant health and economic problem. Obesity has been reported to be a major contributor to variety of chronic diseases. Childhood obesity has been rising over the past decades leading to various complications in health. Millions of infants and children undergo surgery every year on various health grounds. The present investigation was undertaken to evaluate the effect of spinal anesthesia of equipotent doses of ropivacaine and bupivacaine on over-weight neonatal rats.
Methods: The Sprague-Dawley rat pups were overfed on high fat diet to induce obesity. Behavioral assessments for sensory and motor blockade was made by evaluating thermal and mechanical withdrawal latencies at various time intervals following intrathecal injections of bupivacaine (5.0 mg·kg^{-1}) and ropivacaine (7.5 mg·kg^{-1}) in P14 rats. Spinal tissue was analyzed for apoptosis by determination of activated caspase-3 using monoclonal anti-activated caspase-3 and Fluoro-Jade C staining. Long-term spinal function in P30 rat pups was evaluated.
Results: Exposure to intrathecal anesthesia in P14 increased thermal and mechanical latencies and was observed to increase apoptosis as presented by increase in activated caspase-3 and Fluro-Jade C positive cells. Significant alterations in spinal function were observed in high fat diet-fed pups as against non-obese control pups that were on standard diet. Bupivacaine produced more pronounced apoptotic effects on P14 pups; ropivacaine however produced long lasting effects as evidenced in motor function tests at P30.
Conclusion: Ropivacaine and bupivacaine induced spinal toxicity that was more pronounced in over-fed rat pups as against normal controls.

* Corresponding author.
E-mail: zhuliping278@gmail.com (L.-P. Zhu).

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PALAVRAS-CHAVE
Bupivacaína;
Ropivacaína;
Anestesia intratecal;
Toxicidade medular;
Obesidade

Efeito de doses equipotentes de bupivacaína e ropivacaína em modelo de roedor neonatal alimentado com dieta rica em gordura

Resumo
Objetivos: O aumento da prevalência da obesidade é um problema sério de saúde e econômico. A obesidade tem sido relatada como um dos principais contribuintes para uma variedade de doenças crônicas. A obesidade infantil tem aumentado nas últimas décadas, levando a várias complicações de saúde. Milhões de bebês e crianças são submetidos à cirurgia todos os anos por diversos motivos de saúde. O presente estudo foi realizado para avaliar o efeito da raquianestesia com doses equipotentes de ropivacaína e bupivacaína em ratos recém-nascidos com sobrepeso.
Métodos: As crias de ratos Sprague-Dawley foram alimentadas em excesso com dieta rica em gordura para induzir obesidade. Avaliações comportamentais para bloqueio sensorial e motor foram feitas através da avaliação das latências de retirada térmicas e mecânicas em vários intervalos de tempo após injeções por via intratecal de bupivacaína (5,0 mg·kg^{-1}) e ropivacaína (7,5 mg·kg^{-1}) em ratos P14. Tecido medular foi analisado para apoptose por determinação da caspase-3 ativada, usando anticorpo monoclonal anti-caspase 3 ativada e ecoloração com Fluoro-Jade C. A função da coluna vertebral a longo prazo em filhotes de ratos P30 foi avaliada.
Resultados: A exposição à anestesia intratecal em P14 aumentou as latências térmicas e mecânicas e observamos aumento da apoptose, como apresentado pelo aumento da caspase-3 ativada e células positivas para Fluro-Jade C. Alterações significativas da função da coluna vertebral foram observadas em filhotes alimentados com dieta rica em gordura versus filhotes controles não-obesos em dieta padrão. Bupivacaína produziu efeitos apoptóticos mais pronunciados sobre os filhotes P14; ropivacaína, entretanto, produziu efeitos duradouros como evidenciado nos testes de função motora em P30.
Conclusão: Ropivacaína e bupivacaína induziram toxicidade medular mais pronunciada nos filhotes de ratos sobrealimentados que nos controles normais.

Introduction

Childhood obesity is one of the most serious public health challenges of the 21st century. The prevalence of childhood obesity has increased at an alarming rate. Obesity affects around 1.5 billion people around the globe today[1,2] of which 200 million are children.[3] Obese and over-weight children have higher chances of developing obesity-related complications.[4,5]

Obesity has also been reported to be associated with several surgical pathologies.[6] The physiological changes in obesity markedly affect distribution, binding, and elimination of anesthetic drugs[7–9] and severe adverse reactions could possibly result if drug dosing is based only on the actual body weight of the patient.

Regional anesthesia as combined spinal–epidural anesthesia or integrated epidural and light general anesthesia are often employed in obese patients to lessen the risks related to airway control and postoperative respiratory depressions that are induced by general anesthetics or opioids used in treatment of pain.[10]

Bupivacaine is used as a local anesthetic that is employed in nerve block, epidural, and intrathecal anesthesia and is often administered to control pain before, during and after spinal surgery.[11] Although extensively used in pain control, bupivacaine has been reported to be cardiotoxic, neurotoxic, and is the most myotoxic of the local anesthetics.[12]

Ropivacaine, an aminoamide derivative is a local anesthetic drug that is structurally related to mepivacaine and bupivacaine, and has therapeutic properties similar to those of bupivacaine but associated with less motor blockade and toxicity.[13] In clinical studies, ropivacaine appears to be suitable for both epidural and regional anesthesia.[14] The present study was undertaken to study the effects of bupivacaine and ropivacaine in over-weight/obese neonatal rats.

Materials and methods

Animals

This study was approved by the Medical Ethics Committee of the First People's Hospital of Jining, institutional animal care committee and was performed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals. The pregnant female Sprague-Dawley rats (Guangdong Medical Laboratory Animal Co., China) were used. The rats were housed in a room on a 12 h light/dark cycle with free access to water. The animals were housed individually in separate cages and monitored closely for the day of birth, which was considered as postnatal day 0 (P0). The rat pups (male and female) were kept in cages in a room on a 12 h light/dark cycle with free access to water with their littermates till P5. Neonatal overfeeding was done to induce

obesity by reducing litter size to 3 pups per litter (small litter, SL) on P6, while normal litters (NL) were culled to 10 pups per litter. The pups of SL were provided access to high fat diet from P6 till P21. High fat diet was prepared with butter, milk powder, wheat flour and sugar in equal proportions each. The rats were fed with high fat diet at 4 g/day along with standard rat chow. The animals of both SL ($n = 36$) and NL ($n = 36$) were monitored carefully. On P14, the rats were grouped separately for experiments. The NL control pups (NLC) received no anesthesia and were fed on normal standard diet and SL control pups (SLC) received no anesthesia but were fed on high fat diet. The treatment groups NL and SL pups were induced with intrathecal bupivacaine (NLB and SLB) and ropivacaine (NLR and SLR) on P14. On P14, the body weights of NL pups were 23–28 g and that of SL pups were between 26 and 34 g.

Intrathecal injections of bupivacaine and ropivacaine

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. A constant concentration of bupivacaine was injecting varying volumes scaled to the rat pup's body weight at a dose of 5.0 mg·kg^{-1} b.wt. Ropivacaine at 7.5 mg·kg^{-1} b.wt. was administered.

Behavioral assessments for sensory and motor blockade

The P14 rats underwent baseline measurement of hind paw thermal withdrawal latencies immediately before spinal injection. Blockade of thermal nociception was assessed using a modified hot plate test as described previously.[15,16] Hind paws were exposed in sequence (left then right) to a hot plate (model 39D hot plate analgesia meter; IITC Inc., Woodland Hills, CA, USA) at 52 °C for P14. 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.

Blockade of mechanical nociception was assessed by hind paw withdrawal using von Frey filaments. The von Frey filaments apply logarithmically increasing pressure. Pups were lightly restrained on a flat surface and well calibrated von Frey hairs device (electronic von Frey device, Stoelting, Wood Dale, IL, USA) that deliver increasing mechanical stimuli were applied to the dorsal surface of the hindpaw of the pups, five times with one second intervals.[17] The number of evoked withdrawal responses to each stimulus of increasing intensity was recorded until a given stimulus evoked five responses or until a suprathreshold cut-off pressure was reached.[17] Mechanical withdrawal thresholds were recorded at baseline and every 10 min for at least 40 min after the intrathecal injection and until full recovery was observed. In both the thermal and mechanical withdrawal tests, animals were observed for the possibility of exhibiting motor blockade without sensory blockade based on 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).

Motor behavior on postnatal day 30

Motor impairment was assessed on the P30 rats that had undergone spinal bupivacaine or ropivacaine injections on P14. These rats were introduced to a Dual species Economex Rotarod (Columbus Instruments, Columbus, OH, USA) using a spindle rotating at 10 rotations per minute.[18] 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.

Euthanasia and perfusion

The animals were euthanized with intraperitoneal injection of sodium pentobarbital (100 mg·kg^{-1}) and perfused transcardially with saline followed by 4% paraformaldehyde 6 h after the treatment periods ended. The spinal anesthesia groups receiving bupivacaine or ropivocaine were perfused 6 h following initiation of the anesthetic intervention. After gentle dissection of the spinal cord, the tissues were stored overnight at 4 °C in 4% paraformaldehyde and then transferred to a 30% sucrose solution at 4 °C until sectioning. The transverse sections of lumbosacral spinal cord (7 and 14 μm) were cut using a cryostat, fixed on slides and stored at −30 °C.

Analysis of activated caspase-3

To assess apoptosis, the spinal tissue obtained from P14 animals 6 h following intrathecal injection of anesthesia was stained for activated caspase-3. The slides were incubated for 10 min in 3% peroxidase, blocked with 0.3% Triton X-100 and 5% normal goat serum in Tris-buffered saline for 1 h at room temperature, followed by incubation with rabbit monoclonal anti-activated caspase 3 (1:100; Cell Signaling, Danvers, MA, USA) overnight at 4 °C. Biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) was applied at 1:250 for 30 min at room temperature. The slides were further incubated with avidin–biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min and developed with 3,3′-diaminobenzidine (DAB) for 8 min. The slides were counterstained with hematoxylin, dehydrated and observed.

Fluoro-Jade C staining

Fluoro-Jade C staining was performed in 14 μm sections of spinal cord that were obtained 6 h following intrathecal injections. The staining was done using Fluoro-Jade C staining kit (Biosensis, USA) following the manufacturer's instructions. Fluoro-Jade C immunfluorescent positive cells were counted under the appropriate wavelength fluorescent microscopy.

Statistical analysis

Data are expressed as mean ± SD from at least six independent experiments. ANOVA (One-way analysis of variance) at $p < 0.05$ was considered statistically significant. The statistical analyses were performed using SPSS software (version 17.0).

Results

Animal response to thermal stimulus

Hind paw thermal withdrawal latencies were determined for P14 (Fig. 1B) rats in groups receiving spinal bupivacaine and ropivacaine. Both 0.5 mg·kg^{-1} bupivacaine and 0.75 mg·kg^{-1} ropivacaine doses produced dense thermal nociceptive blockade at the first measurement at 10 min following injection compared to control and remained dense at 20 min. The thermal withdrawal latencies remained significantly greater than control values at 30 min for ropivacaine than bupivacaine. However thermal withdrawal latencies remained higher on bupivacaine injection than control at 30 and 40 min (Fig. 1A). The latencies were observed to be markedly higher in the over-weight pups administered with bupivacaine or ropivacaine compared to non-over-fed rat pups. The control over-weight rat pups exhibited latencies higher than normal litter control pups (Fig. 1B).

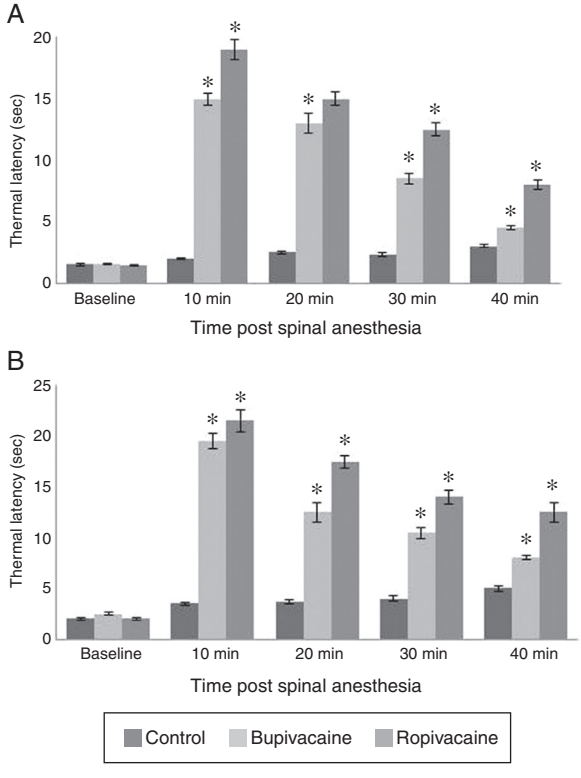

Figure 1 (A) Thermal latency of P14 normal litter rat pups following spinal anesthesia. (B) Thermal latency of P14 small litter rat pups following spinal anesthesia. Values are represented as mean ± SD ($n = 6$). *Represents statistical significance at $p < 0.05$ compared against control as determined by ANOVA.

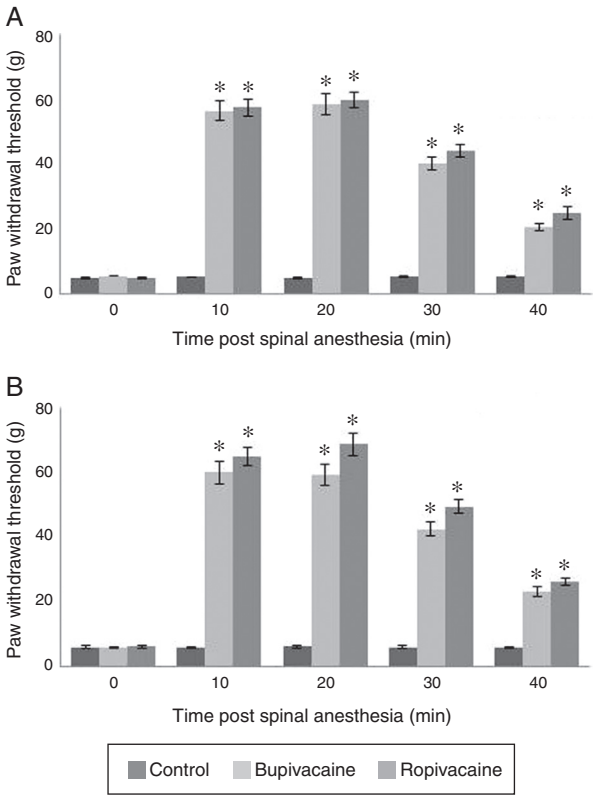

Figure 2 (A) Paw withdrawal threshold of P14 normal litter rat pups following spinal anesthesia determined using von Frey filaments. (B) Paw withdrawal threshold of P14 small litter rat pups following spinal anesthesia determined using von Frey filaments. Values are represented as mean ± SD ($n = 6$). *Represents statistical significance at $p < 0.05$ compared against control as determined by ANOVA.

Response to mechanical stimulus

Withdrawal responses to von Frey filaments applied to the hindpaws are shown in Fig. 2(A and B) for P14, rats in groups receiving spinal bupivacaine and ropivacaine. The threshold values remain higher following intrathecal injections compared to control. The threshold reached a maximum between 5 and 20 min following injections in pups that received ropivacaine and bupivacaine. In the over-fed rat pups the threshold remained higher than control at 30–40 min as compared against the NLB and NLR rat pups.

Table 1 Hind leg motor response of P14 rats after spinal anesthesia.

Rat pup group	Time (min)						
	0	5	10	20	30	40	50
NLC	4	4	4	4	4	4	4
NLB	0	0	0	2	2	4	4
NLR	0	0	0	1	2	4	4
SLC	4	4	4	4	4	4	4
SLB	0	0	0	0	2	2	4
SLR	0	0	0	0	1	1	2

Values are represented as mean, $n = 6$.
Scoring was 0–4 points, based on the sum of ratings for right and left sides.
The score for each hind leg, 0 = no movement, 1 = partial impairment, and 2 = full movement.
NLC, normal litter control pups; NLB, normal litter pups induced with bupivacaine; NLR, normal litter pups induced with ropivacaine; SLC, small litter control pups; SLB, small litter pups induced with bupivacaine; SLR, small litter pups induced with ropivacaine.

Motor block scores

Motor block scores are shown in Table 1. Animals that received neither ropivacaine nor bupivacaine showed no signs of motor impairment. Bupivacaine and ropivacaine produced dense motor block in all animals that recovered almost completely by 40 min in all NL animals. Whereas in SL animals that received intrathecal injections presented blocks in SLR and SLB even at 40 min. SLR and NLR pups exhibited motor blocks more than SLB and NLB respectively at 40 min (Table 1). Control animals that received no anesthesia responded to pinch with forceps on the skin of the back with a startled jerks and exhibited escape behaviors. All animals that received spinal injections of bupivacaine and ropivacaine exhibited no behavioral response to pinch over the skin of the back at lumbar and lower thoracic levels, but showed slight withdrawal behaviors to pinch at upper thoracic levels and on the forepaws following 10–15 min after injections. Animals of NL and SL groups that received either bupivacaine or ropivacaine exhibited similar responses to pinch.

Motor performance in adult rats with postnatal anesthetic exposures

P30 rats that were exposed to intrathecal injections of ropivacaine and bupivacaine at P14 were tested for motor performance using the Rota Rod apparatus. Differences observed between the groups were not significant although the control group that received neither ropivacaine nor bupivacaine exhibited higher values than the other groups (Fig. 3).

Cleaved caspase-3 and Fluoro-Jade C staining for apoptotic neurodegeneration

The number of activated caspase-3 and Fluoro-Jade C positive cells in the lumbar spinal cord of the P14 rat pups

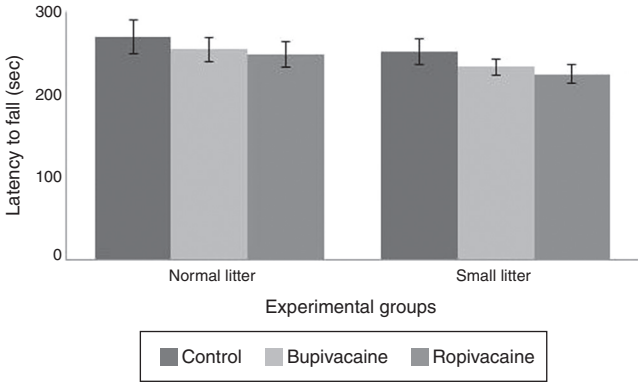

Figure 3 Latency of falling of P30 rat pups following spinal anesthesia on P14. Values are represented as mean ± SD ($n = 6$).

that received intrathecal injections were observed to be markedly ($p < 0.05$) higher as against normal control pups. The apoptotic counts were higher in the rats receiving bupivacaine than ropivacaine. The counts though higher are not markedly different. In SLB, the apoptotic cell counts were slightly more than NLB. Similar results as in bupivacaine administration were observed between SLR and NLR (Figs. 4 and 5).

Discussion

Anesthetics induce an unconscious state to devoid pain and awareness, and millions of children each year are exposed to anesthesia as a part of their medical care. Nevertheless, the safety of anesthesia in pediatric population is a matter of concern and previous retrospective studies have reported a correlation between anesthesia exposure and learning disabilities.[19,20] In addition, various animal models have shown that early anesthetic exposure results in significant long-term behavioral deficits.[21–23]

Bupivacaine is a commonly used anesthetic for pain control in low back pain and spinal surgery. Bupivacaine binds to the intracellular sodium ions and blocks sodium influx into nerve cells which prevents depolarization.

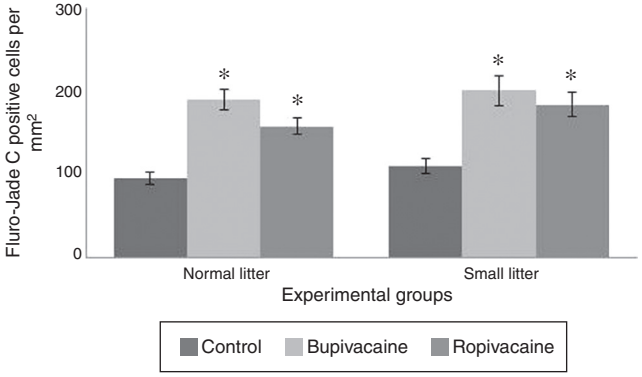

Figure 4 Fluro-Jade C positive cells in the spinal cord sections of P14 rat pups following spinal anesthesia. Values are represented as mean ± SD ($n = 6$). *Represents statistical significance at $p < 0.05$ compared against respective controls as determined by ANOVA.

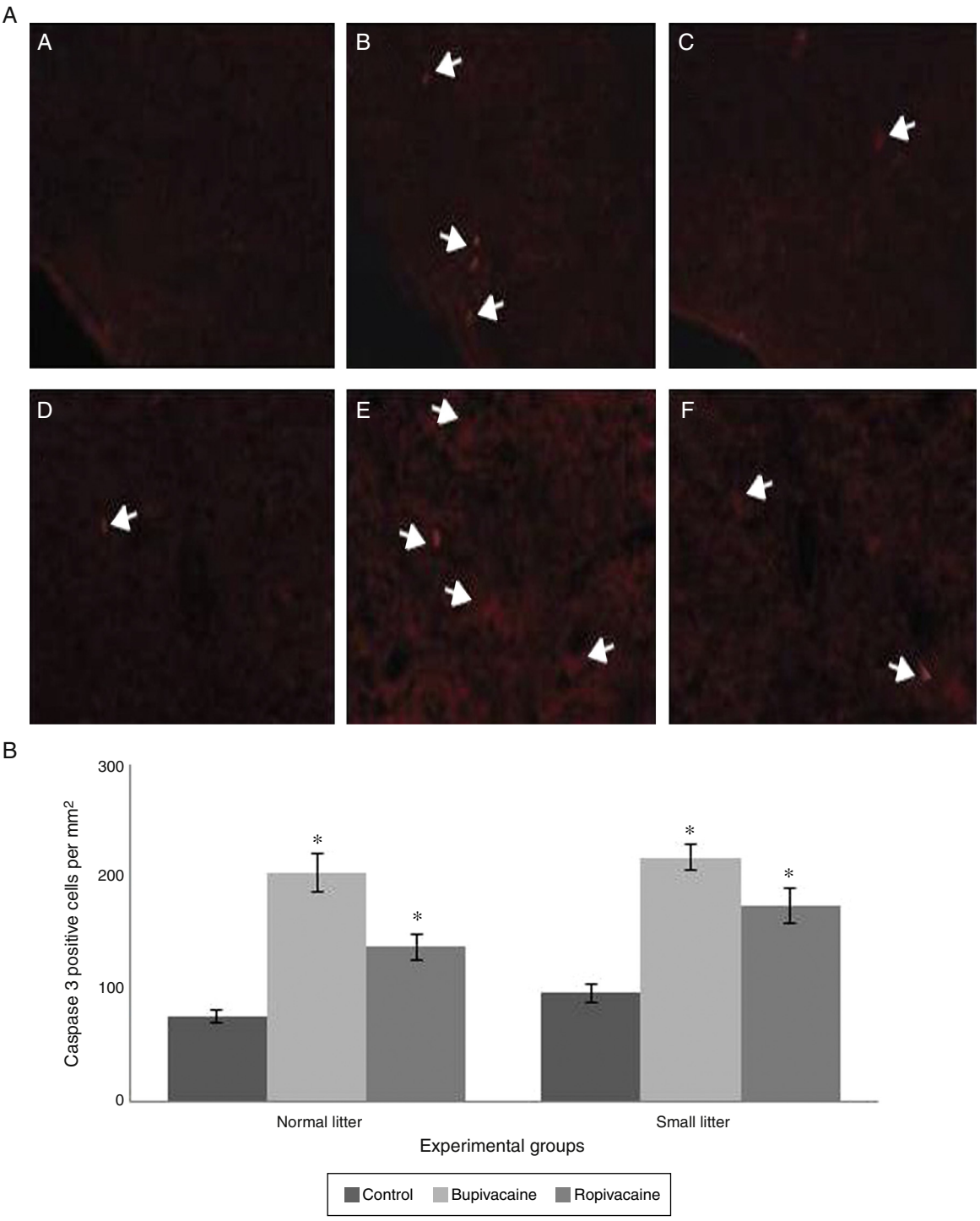

Figure 5 Caspase-3 positive cells in the spinal cord sections of P14 rat pups following spinal anesthesia. Spinal cord sections revealing caspase-3 positive cells. (A) Spinal section of normal litter control pups; (B) spinal section of normal litter pups induced with bupivacaine; (C) spinal section of normal litter pups induced with ropivacaine; (D) spinal section of small litter control pups; (E) spinal section of small litter pups induced with bupivacaine; (F) spinal section of small litter pups induced with ropivacaine. Number of caspase-3 positive cells in spinal cord sections. Values are represented as mean $\pm$ SD ($n = 6$). *Represents statistical significance at $p < 0.05$ compared against respective controls as determined by ANOVA.

In addition, bupivacaine is also a potent uncoupler of mitochondrial oxidative phosphorylation and therefore may induce apoptosis.[24] Indeed, a previous study demonstrated that a fraction of cell death caused by bupivacaine is via apoptosis, although the main mechanism is through necrosis.[25] Ropivacaine is a long-acting regional anesthetic that is structurally related to bupivacaine. Ropivacaine was generally well tolerated in pediatric patients aged from

1 month to 15 years regardless of the route of administration. The overall incidence of adverse events associated with ropivacaine appeared to be low, with nausea and/or vomiting occurring most frequently.[26]

One of the important properties of a long-acting local anesthetic is to reversibly inhibit the nerve impulses, thus causing a prolonged sensory or motor blockade appropriate for anesthesia in different types of surgeries.[27] The results of the assessment of sensory and motor blocks in rat pups following intrathecal anesthesia of ropivacaine or bupivacaine suggests that ropivacaine induced sensory and motor blocks for a longer duration than bupivacaine. Weight of the animal was found to influence the degree of sensory and motor blocks as well. The over-fed mildly obese rat pups exhibited higher thermal and mechanical latencies as against the normally fed rats pups induced with intrathecal injections. However the influence of weight was observed not to effect on the motor performance of the rats at P30. The results of the rotarod experiments suggest no significant variations on the performance of the rats between the various groups. The thermal and mechanical latencies were also observed to fade away by 40–50 min after injections.

Previous studies have investigated the effect of obesity on spinal anesthesia, but the results have not been consistent.[28–35] The observed results differed according to the baricity of the local anesthetics and also the variable chosen for comparison between obese and non-obese patients. Studies have reported positive correlation between obesity and sensory block level.[28–31,35] Thus in our study the changes in blockades and apoptosis levels suggest positive relationship between obesity and sensory blocks. Though the animals of the SL were not designated as obese, the difference in weight between the NL groups did influence to a certain extend the outcome of the blockades following anesthesia. The equipotent doses of ropivacaine and bupivacaine presented variations in the intensity and duration of blocks between the SL and NL nevertheless ropivacaine did not cause vast alterations between the groups. The neurotoxic effects of bupivacaine and ropivacaine have been reported in neonatal rats, in our study at P30 no significant changes were seen in the performance of the pups of both SL and NL, suggesting that the drugs did not cause long-term alterations.

The neurotoxic effect of local anesthetics on the spinal cord has been studied extensively in adult rats. 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.[18]

Cleaved caspase-3 serves as a marker for apoptosis in the detection of anesthetic-induced developmental neurotoxicity. Our observations in neonatal rats have demonstrated that intrathecal bupivacaine and ropivacaine had a similar morphological and apoptotic impact profile. The number of apoptotic cells was higher than control. Bupivacaine however induced much raised levels of caspase-3 positive cells as against ropivacaine. The extent of neurodegeneration as measured by Fluro-Jade C staining was also in line with the above results. The number of Fluro-Jade C positive cells in the spinal sections of the P14 rats following spinal anesthesia was markedly higher than control pups. The results suggest bupivacaine as more neurotoxic than ropivacaine and body weight did/does influences the extent of neurotoxicity and apoptosis. Although ropivacaine was found to produce long lasting blocks, it induced neuronal toxicity to some extent.

The observations on the developmental neurotoxicity of anesthetic agents in laboratory animals question their safety in clinical practices with raising over-weight of pediatric population. Further studies could be elaborated on having thorough investigations on the mechanisms involved and measures to reduce undesirable side effects.

Conflicts of interest

The authors declare no conflicts of interest.

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