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Neurotoxic effects of local anesthetics on the mouse neuroblastoma NB2a cell line

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Abstract

Local anesthetics are used clinically for peripheral nerve blocks, epidural anesthesia, spinal anesthesia and pain management; large concentrations, continuous application and long exposure time can cause neurotoxicity. The mechanism of neurotoxicity caused by local anesthetics is unclear. Neurite outgrowth and apoptosis can be used to evaluate neurotoxic effects. Mouse neuroblastoma cells were induced to differentiate and generate neurites in the presence of local anesthetics. The culture medium was removed and replaced with serum-free medium plus 20 µl combinations of epidermal growth factor and fibroblast growth factor containing tetracaine, prilocaine, lidocaine or procaine at concentrations of 1, 10, 25, or 100 µl prior to neurite measurement. Cell viability, iNOS, eNOS and apoptosis were evaluated. Local anesthetics produced toxic effects by neurite inhibition at low concentrations and by apoptosis at high concentrations. There was an inverse relation between local anesthetic concentrations and cell viability. Comparison of different local anesthetics showed toxicity, as assessed by cell viability and apoptotic potency, in the following order: tetracaine > prilocaine > lidocaine > procaine. Procaine was the least neurotoxic local anesthetic and because it is short-acting, may be preferred for pain prevention during short procedures.

Key words: anesthetics, apoptosis, cell viability, neurites, neurotoxicity

Local anesthetics (LAs) are used clinically for peripheral nerve blocks, epidural anesthesia, spinal anesthesia and pain management (Kuthiala and Chaudhary 2011, Perez-Castro et al. 2009, Zhang et al. 2014). These agents inhibit Na^+, Ca^{2+} and K^+ channels (Perez-Castro et al. 2009, Zhang et al. 2014). There are two kinds of LAs: esters and amides. The esters are short-acting and the amides are long-acting. The most important complication of LA administration is neurologic injury including transient neurological symptoms, persistent lumbosacral neuropathy and cauda equina syndrome (Perez-Castro et al. 2009, Werdehausen et al. 2009, Yamashita et al. 2003). It has been suggested that these complications may be due to the use of high concentrations of or extended exposure to LAs (Perez-Castro et al. 2009, Sekimoto et al. 2006, Werdehausen et al. 2009). We compared the neurotoxicity of four different LAs at different concentrations on the mouse neuroblastoma NB2a cell line. We examined neurite inhibition (NI), cell viability and apoptosis.

Material and methods

Mouse NB2a neuroblastoma cells were obtained from the European Collection of Cell Cultures (ECACC; cell line: 89121404). Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO). Tissue culture flasks and plates were obtained from Falcon/Fred Baker (Runcorn, Cheshire, UK), and gentamicin (Genta®; 20 mg

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ampule) was purchased from I. Ethem (Istanbul, Turkey).

Cell culture

NB2a cells were grown in culture flasks in a humidified incubator at 37° C with 5% CO_2. The NB2a cells were grown in 5 ml high glucose Dulbecco's modified Eagle medium (DMEM) with glutamax-1 and supplemented with 5% (v/v) horse serum, 5% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/m streptomycin and 25 µg/ml gentamicin (Smith et al. 1997).

Neurite outgrowth

NB2a cells were seeded onto 24-well culture plates at a density of 15,000 cells/ml. After 24 h, the cells were induced to differentiate and generate neurites in the presence of LAs using the following method. The culture medium was removed from the wells and replaced with serum-free medium plus 20 µl combinations of epidermal growth factor (EGF) and fibroblast growth factor (FGF) containing tetracaine, prilocaine, lidocaine or procaine at concentrations of 1, 10, 25, or 100 µl. Ethanol, which was used as a vehicle, served as a positive control. The cells were incubated for an additional 24 or 48 h. Cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature, stained for 3 min with Coomassie blue (0.6% Coomassie brilliant blue G in 10% acetic acid, 10% methanol and 80% PBS), then washed with PBS. The samples were photographed through the microscope and transferred to a computer; morphometric parameters were measured by three blinded observers) using an Olympus BX40 light microscope (Olympus, Tokyo, Japan) with a video camera (JVC-TK-C 601; Tokyo, Japan) for digital imaging. Images were analyzed using an Image-Pro Plus image analyzer (5.1.259; Bioscience Technology, Bethesda, MD). Subsequently, 10 fields that contained approximately 10 cells each were selected for measurement for each LA group. A software algorithm was designed using the image analyzer functions of the microscope to enable automatic measurement of total neurite length (in pixels) for the cells in a given field and to express the results as the average neurite length per cell (Smith et al. 1997).

Cell viability

To determine cell viability, 6-well plates were seeded with 5 x 10^4 cells in 1,500 µl medium, and 1, 10, 25, or 100 µl of a given LA was added. After 24 h, trypan blue (0.4%) (Ye et al. 2014) was added to the cell suspensions and the stained cells were counted in four different fields of a Thoma cell counting chamber. The ratio of viable cells to dead cells was calculated.

Differentiation, oxidative stress and apoptosis

We used the oxidative stress markers, endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS), to detect effects of LA on neuroblastoma cells. After administration of serum-free medium plus 20 µl combinations of epidermal growth factor (EGF) and fibroblast growth factor, neuronal differentiation was assessed using a Mouse Rat Neural Stem cell Functional İdentification Kit (SC013; RD Systems, Minneapolis, MN) for nestin, tubulin, glial fibrillary acidic protein (GFAP) and oligodendrocyte marker O4.

To investigate neuronal differentiation, the cells were fixed in 4% paraformaldehyde (1.04004; Merck, Darmstadt, Germany) for 30 min, then Mouse Rat Neural Stem cell Functional İdentification Kit (SC013; RD Systems, Minneapolis, MN) was used according to the manufacturer's instructions. For immunohistochemical staining, after application of the LA, cells were fixed with 4% paraformaldehyde for 30 min and washed in phosphate buffered saline solution (PBS, 00-3002, İnvitrogen, Camarillo, CA) three times for 5 min each time. Permeabilization was effected using 0.1% Triton X-100 (A4975; AppliChem, Darmstadt, Germany) in PBS at 4^0 C for 15 min. Cells were washed in PBS, were incubated in 3% H_2O_2 solution for 5 min to block endogenous peroxidase activity, washed with PBS, and incubated for 18 h at 4° C with monoclonal anti-endothelial nitric oxide synthase (eNOS; rabbit polyclonal antibody, pAb; RB-1711-P1; Neomarkers, Fremont, CA), anti-inducible nitric oxide synthase (iNOS; rabbit pAb, RB-1605-P; Neomarkers). The cells were washed three times for 5 min each time in PBS, incubated with biotinylated goat immunoglobulin G (IgG) anti-rabbit IgG, then with horseradish peroxidase-conjugated streptavidin for 30 min each (peroxidase-based LSAB 2 kit; Dako). After washing three times for 5 min each time with PBS, cells were incubated in 3, 3' diaminobenzidine (DAB; Dako) for 5 min for immunolabeling and subsequently stained with Mayer's hematoxylin. Cells were covered with mounting medium and viewed using an Olympus light microscope. Control samples were processed identically except that the primary antibody was omitted. Two observers, who were blinded to the

identity of the sections, evaluated the staining independently. Scoring was semiquantitative according to intensity of the staining: 0, no staining; 1, weak staining; 2, moderate staining; 3, moderate − strong staining; 4, strong staining; 5, very strong staining (Toprak et al. 2002).

To detect apoptosis in situ, we used the terminal deoxynucleotidyl transferase-biotin nick end-labeling (TUNEL) method. Nuclear DNA fragmentation is an important morphological characteristic of apoptosis and can be detected in the cells by TUNEL. We used a commercial kit (DeadEnd Colorimetric TUNEL system, G7130; Promega, Madison, WI) according to the manufacturer's instructions. Briefly, after proteinase K treatment for 10 min, the cells were incubated at 37° C with terminal deoxynucleotidyl transferase (TdT) for 60 min. TdT was omitted prior to an end of reactions as the TUNEL negative staining control, (Gürpınar et al. 2012). TUNEL staining was assessed by a blinded observer, who counted TUNEL positive cells in selected areas; the percentage of apoptotic cells then was calculated.

Statistical analysis

The data are expressed as means ± SD and were analyzed using repeated-measures ANOVA. The Tukey-Kramer multiple comparisons test was used to determine differences among means. Values for $p \leq 0.05$ were considered significant.

Results

We used NI, cell viability and apoptosis to assess neurotoxicity caused by four LAs. The mouse neuroblastoma cell line reached confluency after 7 days, and neurite outgrowth was observed after application of neuronal differentiation factors that stimulate neurite outgrowth, i.e., combinations of 20 µl EGF and 20 µl FGF for each well. Neurite outgrowth and neuronal differentiation were observed using phase contrast microscopy (Fig. 1). By the end of day 7, cells were stained immunocytochemically for the neuronal differentiation markers, nestin and tubulin. Little glial immunostainingwas observed, however, by glial fibrillary acidic protein (GFAP) for astrocytes or oligodendrocyte marker O4 for oligodendrocytes (Fig. 2).

We evaluated the toxic effects of varying concentrations of four LAs during NB2a cell differentiation. Neurite outgrowth was investigated using Coomassie blue and cell viability was investigated using trypan blue (Fig. 1). For cell viability, all groups were compared to one another to determine concentration dependence. For groups treated with 1 µl LA, we found statistically significant differences between the procaine and prilocaine groups ($p < 0.05$), and between the procaine and tetracaine groups ($p < 0.01$). For 1 µl LA treated groups, tetracaine was the most neurotoxic. For the groups treated with 10 µl LA, we found statistically significant differences between the procaine and tetracaine groups ($p < 0.001$), procaine and prilocaine groups ($p < 0.01$), lidocaine and prilocaine groups ($p < 0.05$), and lidocaine and tetracaine groups ($p < 0.01$). For the groups treated with 10 µl LA, tetracaine was the most neurotoxic and procaine was the least toxic. For the groups treated with 25 µl LA, differences among groups were not statistically significant. For the groups treated with 100 µl LA, statistically significant difference was detected only between the procaine and tetracaine groups ($p < 0.01$). For the 100 µl LA treated groups, tetracaine was the most neurotoxic and procaine was the least neurotoxic (Fig. 4)

To evaluate the effects of oxidative stress after LA application, we stained cells for i-NOS and e-NOS to detect the presence of free radicals. We also used TUNEL staining to evaluate apoptosis to clarify the relation between oxidative stress and programmed cell death (Fig. 3). We found that the cultured cells were immunopositive for oxidative stress and that the intensity of immunoreactivity was dependent on the levels of toxicity. Immunocytochemistry showed that apoptosis was related to oxidative stress (Fig. 3).

We demonstrated the relation between oxidative stress and toxicity-induced apoptosis using morphometric measurements. We observed a small

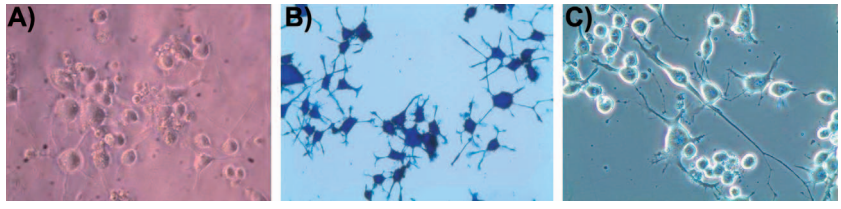

Fig. 1. A) Neurite differentiation after application of 20 µl combinations of EGF and FGF. B) Cell viability. Trypan blue. C) Neurite outgrowth. Coomassie blue. x 400.

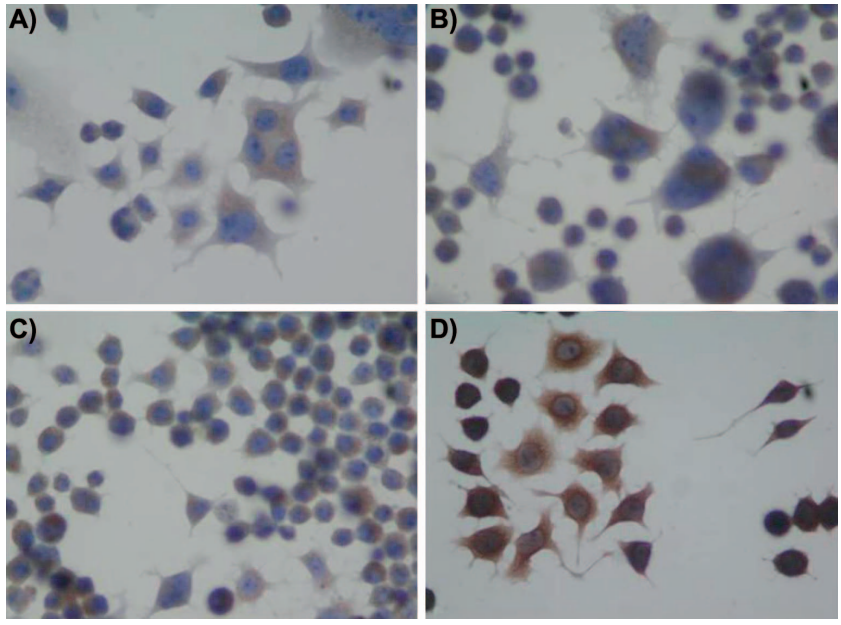

Fig. 2. Staining of cells with GFAP (A), oligodendrocyte (B), nestin (C) and tubulin (D). x 400.

amount of NI in the PBS treated control group. The basal level of e-NOS was greater than that of i-NOS. TUNEL positive apoptotic cells were detected rarely in any field. LA treatment significantly increased all parameters (i-NOS, e-NOS, TUNEL and NI). All parameters were compared among all groups. For NI and i-NOS, a significant difference was detected only between the procaine and tetracaine groups ($p < 0.05$ and $p < 0.01$ for NI and i-NOS, respectively). NI and i-NOS staining were greatest in the tetracaine treated group and least in the procaine treated group. There was no significantly difference, however, between any groups for e-NOS (Fig. 5).

There were significant differences in TUNEL staining among the procaine and tetracaine groups ($p < 0.001$), procaine and prilocaine groups ($p < 0.01$) and lidocaine and tetracaine groups ($p < 0.05$). TUNEL staining was greatest in tetracaine group and it was least in procaine group (Fig. 5).

NOS expression increased with TUNEL staining and NI, which suggests that cells responded to increased oxidative stress by apoptosis and NI.

LAs exerted their toxic effects by neurite inhibition at low concentrations and by apoptosis at high concentrations. There was an inverse relation between LA concentration and cell viability. Comparison of the different LAs showed toxicity, as assessed by cell viability and apoptotic potency, in the following order: tetracaine > prilocaine > lidocaine > procaine (Figs. 4, 5).

Discussion

LAs are effective for pain relief, but they have potential side effects (Kuthiala and Chaudhary 2011). These agents inhibit Na^+, Ca^{2+} and K^+ ion channels (Kasaba et al. 2003, Kuthiala and Chaudhary 2011, Perez-Castro et al. 2009, Zhang et al. 2014). LAs also can affect N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Zhang et al. 2014). It has been suggested that high concentration, and long exposure time to LAs can cause neurotoxicity (Perez-Castro et al. 2009, Sekimoto

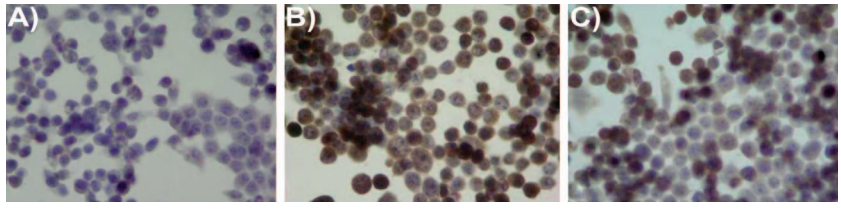

Fig. 3. i-NOS (A), e-NOS (B) and TUNEL (C) staining to evaluate oxidative stress, free radicals and apoptosis, respectively. x 400.

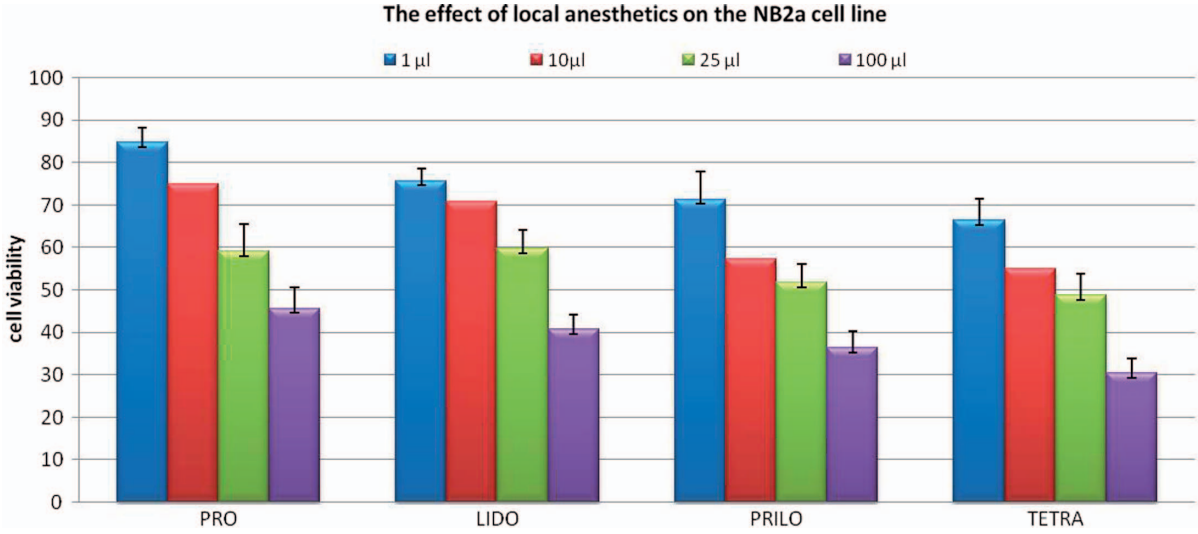

Fig. 4. Inverse relation between cell viability and drug concentrations for each LA tested. PRO, procaine; LIDO, lidocaine; PRILO, prilocaine; TETRA, tetracaine.

et al. 2006, Werdehausen et al. 2009); conversely, Pollock et al. (2002) reported that high concentration or hyperosmolarity were not contributing factors. Lipophilic properties of LA and lithotomy position of the patient, e.g., intrathecal administration of LA during knee surgery, also have been implicated in neurotoxicity (Eng et al. 2014, Werdehausen et al. 2009).

We investigated both the ester (tetracaine and procaine) and amide type (lidocaine and prilocaine) LAs; we found no relation between neurotoxicity and the ester amide type. Both ester and amide types gave similar results.

The mechanism by which LAs induce neurotoxicity remains unclear. The literature contains many clinical and laboratory reports concerning the neurotoxic effects of different LAs that were evaluated by neurite outgrowth, apoptosis, and clinical and histopathological changes (Johnson et al. 2004, Kamiya et al. 2005, Kasaba et al. 2003, Kishimoto et al. 2002, Lee et al. 2009, Saito et al. 2001, Sekimoto et al. 2006, Takenami et al. 2009).

Neurite outgrowth is a basic neuronal function that depends on axonal transport, microtubule recombination and neurofilament proteins. Biological and toxic environmental substances can inhibit neurite outgrowth; therefore, monitoring neurite growth can be used to investigate the neurotoxic activity of LAs (Axelrad et al. 2003). Kasaba et al. (2003) demonstrated neurotoxic effects of LA in

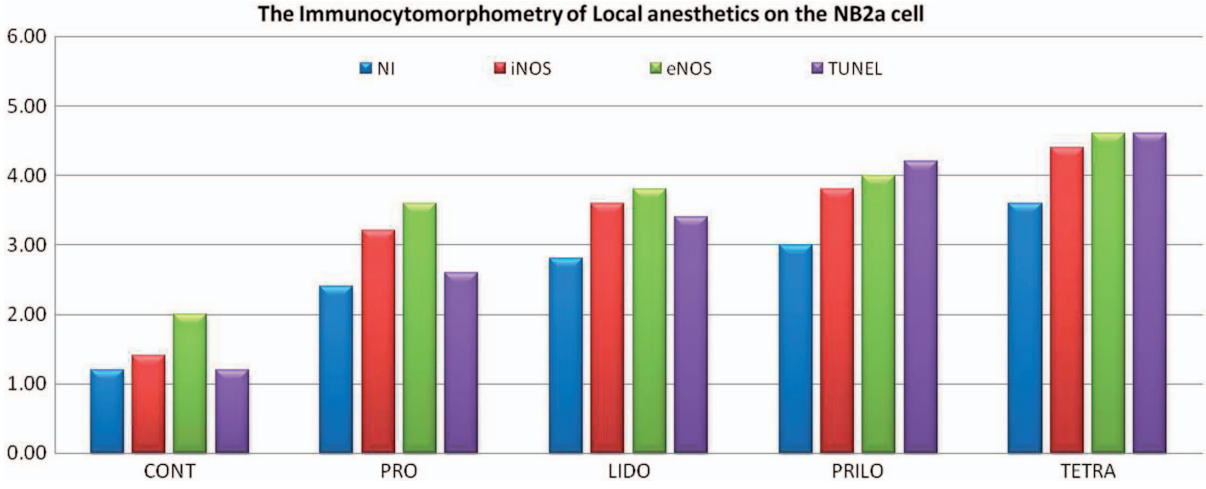

Fig. 5. Immunocytomorphometry of LAs. i-NOS and e-NOS show oxidative stress. TUNEL shows apoptosis. CONT, control; NI, neurite inhibition; PRO, procaine; LIDO, lidocaine; PRILO, prilocaine; TETRA, tetracaine.

vitro as manifested by the collapse of growth cones and neurites in cultured neurons exposed to LAs. These investigators reported the order of neurotoxicity of LAs to be: procaine = mepivacaine < ropivacaine = bupivacaine < lidocaine < tetracaine; they did not evaluate prilocaine. Similarly, Saito et al. (2001) evaluated the neurotoxicity of tetracaine on growth cones and neurites of growing neurons and concluded that short term exposure produced irreversible changes in growing neurons. We observed that all LAs caused neurite inhibition in a concentration-dependent manner; tetracaine caused the most and procaine the least neurite inhibition (Fig. 5).

Apoptosis also can be used to evaluate neurotoxic effects. LAs increase mitochondrial membrane permeability and decrease ATP production by uncoupling oxidative phosphorylation and inhibiting the mitochondrial respiratory chain (Perez-Castro et al. 2009). In addition, some reports indicate that LAs cause apoptosis by DNA mutation, caspase activation, increased cytochrome c levels in the cytoplasm and loss of phosphatidylserine membrane asymmetry (Johnson et al. 2004, Kamiya et al. 2005, Lee et al. 2009). Werdehausen et al. (2009) compared LA induced apoptosis with respect to chemical structure (ester or amide type) in human neurons and reported that LAs cause apoptosis in the following order of potency: tetracaine > bupivacaine > prilocaine = mepivacaine = ropivacaine > lidocaine > procaine = artacaine. Lee et al. (2009) concluded that tetracaine induces apoptosis by generating reactive oxygen species in rat cortical astrocytes. Chang et al. (2014) studied the apoptotic effects of LAs in human thyroid cancer cells and reported that lidocaine and bupivacaine caused apoptosis by the mitogen-activated protein kinase pathway. Our results were consistent with those reported by Werdehausen et al. (2009); all LAs caused apoptosis in a concentration-dependent manner. Cell viability was inversely related to LA concentration. Tetracaine caused the most apoptosis, procaine the least.

The neurotoxicity of LAs has been investigated also using animal models to examine clinical and morphological changes (Kishimoto et al. 2002, Lee et al. 2009, Takenami et al. 2009, Yamashita et al. 2003). Yamashita et al. (2003) compared the effects of LAs on the glutamate concentration in cerebrospinal fluid, and on neurologic and histopathologic outcomes in rabbits. These investigators reported that although the histopathological changes were similar after both lidocaine and tetracaine administration, sensory and motor functions were affected adversely in the lidocaine group. Kishimoto et al. (2002) compared spinal neurotoxicity caused by prilocaine and lidocaine in rats and concluded that the functional impairment and morphological damage were equal. Takenami et al. (2009) compared the neurotoxic effects of LAs in rats and reported that procaine caused less histological damage than prilocaine.

The different results reported by different investigators likely are due to differences in experimental materials. For example, neurons in vivo and in vitro exhibit different resistance to toxicity. A significant advantage of studies in vitro is that the concentration of ions in the medium can be controlled. Also, LAs are not exposed to a complex three-dimensional environment and the protective effect of the blood-brain barrier is absent in vitro; therefore, the response of cultured neurons to LA induced toxicity is more precise and may provide more information about possible toxic effects. A significant disadvantage of studies in vitro is that the cells lack exposure to factors produced by other cells, the three-dimensional environment and serum. Neurons also may have less support from astrocytes and oligodendrocytes.

In the clinical environment, LAs are gaining importance for pain prevention during short term procedures such as carpal tunnel release, facet denervation and spinal nerve or peripheral nerve block. We found that procaine was less neurotoxic than lidocaine and prilocaine. We did not investigate ropivacaine, but previous reports demonstrated that ropivacaine is more neurotoxic than procaine (Eng et al. 2014, Kuthiala and Chaudhary 2011).

We believe that all LAs cause neurotoxicity and apoptosis in a concentration-dependent manner. Neurite inhibition can be used as a marker to identify and detect possible damage caused by moderate toxicity. Procaine was the least neurotoxic LA and because it is short-acting, may be preferred for pain prevention during short term procedures.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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