Only tetracaine and not other local anaesthetics induce apoptosis in rat cortical astrocytes

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Background. The potential risks of neurotoxicity due to local anaesthetics after regional anaesthesia have been suggested recently. To evaluate the neurotoxicity of commonly used local anaesthetics, primary cultured rat cortical astrocytes were treated with lidocaine, ropivacaine, bupivacaine, levobupivacaine, and tetracaine.

Methods. Cell death after local anaesthetic treatment was evaluated with a lactate dehydrogenase (LDH) assay. To examine the mechanisms of cell death, reactive oxygen species (ROS) measurement and western blots of poly-ADP ribose polymerase (PARP), procaspase-3, and mitogen-activated protein kinases family members were performed.

Results. Of the local anaesthetics, which were applied at <1 mM for 18 h, only tetracaine significantly increased LDH leakage (P < 0.05) and cell death in a dose- and time-dependent manner. Hoechst 33258–propidium iodide staining and western blots with PARP and procaspase-3 antibodies suggested that tetracaine induced apoptosis. ROS levels increased 2-fold at 30 min after tetracaine treatment compared with the control and then decreased. The antioxidants, N-acetylcysteine and trolox, markedly inhibited tetracaine-induced apoptosis.

Conclusions. Tetracaine induced apoptosis through ROS generation. Further studies focusing on the neurotoxicity of tetracaine are needed.

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Local anaesthetics are widely used in clinical practice. Despite clinical popularity, concerns regarding potential toxicity have been continuously raised. For example, local anaesthetic administration caused histological change in the spinal cord and peripheral nerves. In addition, there have been case reports of a possible increase in the frequency of serious neurological complications after regional anaesthesia.

Astrocytes, the most numerous cells in the central nervous system (CNS), are important in protecting neurones from various external insults and supporting the neurones to function optimally. Astrocytes modulate synaptic transmission and plasticity, guide axonal growth during development, and secrete growth factors to support neuronal survival. They also play a major role in regulating the extracellular ionic environment and protecting neurones from oxidative stress and excitotoxicity. Therefore, dysfunction or loss of astrocytes can lead to neuronal death or dysfunction. Since astrocytes play a central role in maintaining neuronal viability both under normal and pathological conditions, studying the response of astrocytes is essential to understanding the development of neurotoxicity of local anaesthetics.

Although it seems plausible that local anaesthetics may cause dysfunction or loss of astrocytes leading to cytotoxicity, there have been no studies to examine the toxic effects of local anaesthetics on astrocytes, whereas several studies have shown a toxic effect on neuronal cells.

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Therefore, in the present study, the neurotoxicity of commonly used local anaesthetics, lidocaine, ropivacaine, bupivacaine, levobupivacaine, and tetracaine, was evaluated in order to elucidate toxic mechanism in primary cultured cortical astrocytes, and the role of apoptosis.

**Methods**

**Primary astrocyte culture**

All experimental procedures were approved by the Animal Care and Ethics Committee of Seoul National University School of Dentistry and adhered to the ethical guidelines described in the NIH Guide for the Care and Use of Laboratory Animals.

Primary astrocyte cultures were prepared from the whole cortex of 1-day-old Sprague–Dawley rats as described previously. Briefly, brains were removed from the skull and dissected to obtain the cerebral cortex. Dissociated cortical cells were plated at a density of 10⁶ cells per well in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (Gibco), streptomycin 100 μg ml⁻¹, and penicillin 100 units ml⁻¹ (Gibco) and were incubated at 37°C in CO₂ 5%. Experiments were performed when cells were 90–95% confluent. Cortical glial cultures were verified to be at least 95% astrocytes by immunostaining with anti-glial fibrillary acidic protein (Sigma-Aldrich Chemicals, St Louis, MO, USA) as a type I astrocyte marker. The cells were incubated with fresh serum-free DMEM for 24 h before incubation with drugs.

**Reagents**

Local anaesthetics, hydrogen peroxide, and N-acetylcysteine (NAC) were purchased from Sigma-Aldrich Chemicals and were prepared to various concentrations with serum-free media.

**Cell viability and death assay**

Cell viability was measured using the MTT [3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyltetrazolium bromide] assay from Sigma. The cells were seeded into 24-well plates at a concentration of 1×10⁵ cells per well. After exposure to local anaesthetics, DMEM containing 0.5 mg ml⁻¹ MTT was placed into each well of the plates. After incubation for 3 h at 37°C in a CO₂ 5% incubator, the medium was removed and dimethyl sulphoxide 200 μl (Sigma) was added. The absorbance at 570 nm was recorded using a spectrophotometer (Bio-Tek, Winooski, VT, USA), and the results were normalized to the control (100%).

Cell death was assessed by measuring the activity of lactate dehydrogenase (LDH) released into the culture medium. The cells were seeded into 24-well plates at a concentration of 1×10⁵ cells per well. After exposure to local anaesthetics, the decrease in absorbance of nicotinamide-adenine dinucleotide hydrogenase at 340 nm was measured using a spectrophotometer. LDH activity was normalized to control values (0% cell death).

**Hoechst–propidium iodide nuclear staining**

Apoptotic cell death was evaluated by staining the non-viable cells red with propidium iodide (PI) and Hoechst 33258 (Sigma), which stains the nuclei of both live and dead cells blue when examined by fluorescence microscopy. Staining with Hoechst 33258 allows for the discrimination of apoptotic cells on the basis of nuclear morphology and evaluation of membrane integrity. The Hoechst 33258 dye was added to the culture medium at a final concentration of 5 μg ml⁻¹, and the cultured cells were incubated at 37°C for 30 min. The PI 5 μg ml⁻¹ solution was then also added immediately before observation in the fluorescence microscope (BX60; Olympus, Tokyo, Japan) with UV excitation at 300–500 nm.

**Reactive oxygen species measurement**

We determined intracellular reactive oxygen species (ROS) levels by staining cells with 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA; Molecular Probes, Eugene, OR, USA), which was oxidized to highly fluorescent dichlorofluorescein (DCF) by ROS. The cells were loaded with 20 μM H₂DCFDA at 37°C during the last 30 min of anaesthetic treatment. The cells were trypsinized and centrifuged at 300g for 5 min. DCF fluorescence was measured by flow cytometry (Beckton Dickinson, CA, USA).

**Western blot analysis**

After treatment, the cells were washed twice in cold PBS and lysed with buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) supplemented with protease inhibitors. After incubation for 30 min at 4°C, the suspension was centrifuged to obtain the cell extract. Protein content was measured by the Bradford method. An aliquot (30 or 40 μg of protein per lane) was resolved by 10% or 12% SDS–PAGE and blotted to a polyvinyl difluoride transfer membrane. Western immunoblotting was carried out with primary antibodies diluted in 5% non-fat dry milk in Tris-buffered saline and then with a secondary antibody, hydroxyperoxide-linked anti-rabbit or anti-mouse IgG, using ECL systems enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). Polyclonal antibodies to procaspase-3, poly-ADP ribose polymerase (PARP), beta-actin, and mono-phospho-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Statistical analysis**

The Kruskal–Wallis test was used to compare the concentrations of each local anaesthetic in the MTT and LDH
assays. A $P$-value of <0.05 was considered significant. The dose–response data of tetracaine were fitted to a logistic equation yielding the 50% lethal dose (LD$_{50}$). The increase in ROS levels by tetracaine was compared with the control with Student’s $t$-test. All statistical analyses were performed using Prism 4.0 (Graphpad Software Inc., San Diego, CA, USA).

**Results**

On the basis of preliminary results, we confirmed that it was appropriate to choose the concentrations of local anaesthetics up to 1 mM and incubation time up to 18 h to compare the difference of cytotoxicity among local anaesthetics. Eighteen hours after lidocaine, ropivacaine, bupivacaine, levobupivacaine, and tetracaine treatment, tetracaine and bupivacaine induced significant cell death at a concentration of 1 mM compared with the control ($P$$<$0.01, Fig. 1). Moreover, only tetracaine significantly elevated LDH levels at a concentration of 300 $\mu$M ($P$$<$0.01, Fig. 1).

Tetracaine-induced cell death was observed in a dose- and time-dependent manner (Fig. 2A and B). Interestingly, tetracaine 400 $\mu$M evoked LDH release even 1 h after the treatment. The statistically calculated LD$_{50}$ of tetracaine in the primary cultured astrocytes was $\sim$325 $\mu$M (95% confidence interval: 312–338 $\mu$M, Fig. 2C).

Microscopic examination of the untreated and healthy astrocytes (Fig. 3A) showed large cells with a polygonal shape which covered the whole culture plate area without extra space up to 6 h after tetracaine 400 $\mu$M treatment (Fig. 3B). However, astroglial cell death occurred abruptly 9 h after exposure showing the bottom of the culture plate (Fig. 3C). After 18 h, the astrocytes appeared to have condensed nuclei, transformed into an elongated cell shape, and lost their processes. Also, many of the primary cultured astrocytes had detached from the bottom of the culture plate (Fig. 3D). These observations were generally consistent with the assessment of cell death by Hoechst–PI staining (Fig. 4) and with the data from the LDH assay (Fig. 2B). In the presence of tetracaine 400 $\mu$M, the nuclei

![Fig 1](image1.png) **Fig 1** Comparison of cell death induced by lidocaine, ropivacaine, bupivacaine, levobupivacaine, and tetracaine treatment for 18 h in primary cultured astrocytes. Cell death was assessed by LDH assay. Bupivacaine and tetracaine caused significant cell death at a concentration of 1 mM compared with the control. In addition, tetracaine also enhanced significant cell death even at a concentration of 300 $\mu$M compared with the control. The diagrams show the results of five independent experiments. The top of each box represents the median. Error bar indicates inter-quartile ranges of the distribution. *$P$$<$0.01 is statistically different from the control.

![Fig 2](image2.png) **Fig 2** Cytotoxicity of tetracaine with an LDH assay in primary cultured astrocytes. Dose-responsiveness was observed during the 18 h tetracaine treatment (A). The time course of cell death was obtained after treatment with tetracaine 400 $\mu$M (B). The LD$_{50}$ of tetracaine on primary astrocytes was calculated as $\sim$325 $\mu$M (C). The diagrams show the results of five independent experiments. Median and inter-quartile ranges are shown.
of dead cells showed condensed, coalesced, segmented, and fragmented appearances (Fig. 4 C and D). Western blot analyses also showed PARP and procaspase-3 involvement for tetracaine-induced cell death (Fig. 5). Coincident with morphological changes, tetracaine was observed to induce apoptosis in primary cultured astrocytes (Fig. 5).

To elucidate the mechanism of tetracaine-induced apoptosis in astrocytes, ROS involvement in tetracaine-induced apoptosis, which is a commonly known mechanism for cytotoxicity associated with local anaesthetics, was examined. Preliminary data showed that local anaesthetics other than tetracaine had no effect on ROS generation (data are not shown). When ROS production was measured 30 min after treatment with tetracaine 400 μM, ROS reached a maximum elevation of about 2-fold greater than the control, and then decreased.

The addition of the ROS scavengers NAC and trolox inhibited cell death which was induced by tetracaine 400 μM, as measured by MTT assay (Fig. 6).

Discussion
In this study, we showed that tetracaine-induced apoptosis occurred in a dose- and time-dependent manner, which was associated with ROS generation.

In agreement with our data, several animal and in vitro experiments have suggested potential neurotoxic effects associated with the use of tetracaine. Tetracaine induced growth cone collapse and neurite destruction in chick embryo dorsal root ganglia, retinal ganglion cell layers, and sympathetic ganglia. Tetracaine is known to exhibit histological damage of motor neurones in the spinal cord. Intrathecally administered tetracaine, at high concentration, induces histopathological changes in the spinal cord in vivo. However, there is no previous report investigating the effects of tetracaine on primary cortical astrocytes.

Astrocytes are known to maintain local homeostasis in the CNS. They also maintain energy homeostasis by balancing metabolites, such as potassium, and glutamate produced by neuronal activity in the extracellular space. This leads to the speculation that disruption of astrocytes function may mediate harmful effect in the CNS. Astrocytes may also interact via neurotransmitter release from neighbouring neurones and calcium oscillation under dynamic control of neural activity. The communication between neurones and astrocytes indicates that astrocyte dysfunction could lead to neuronal dysfunction and vice versa. Glial dysfunction could affect neuronal survival in primary rat astrocytes cultures by impaired release of neurotrophic factor. Astrocyte damage induced by tetracaine, through
apoptosis as seen in our study, disrupts astrocyte-neurone signalling, leading to neurotoxicity. Previous studies, consistent with our results, demonstrated that tetracaine did not protect against ischaemic insults in rat hippocampal CA1 neurones.\textsuperscript{18} Moreover, it is interesting that cell death occurs after 18 h treatment, despite the fact that tetracaine has a short duration of action. Our results showed that apoptosis of astrocytes was not increased by acute exposure to tetracaine. Our result is consistent with the previous report that neurite degeneration in chick neural tissue occurred in delayed manner 72–98 h after tetracaine treatment.\textsuperscript{12} Lidocaine disrupts axonal transport of neurotrophic factor and induces apoptosis in retinal ganglion cells.\textsuperscript{19} Astrocytes secrete neurotrophic factor to mediate crucial effects for neuroembryogenesis, neuronal survival, and enhance functional integrity of neurones.\textsuperscript{20, 21} Taken together with these findings, the delayed apoptosis in the present study could be the results of either deficiency of neurotrophic factor or impaired neurotrophic factor transport, although further studies are needed to clarify the mechanism of late neuronal apoptosis by tetracaine.

Although the mechanisms involved in the tetracaine-induced apoptosis of astrocytes are still unclear, tetracaine-induced apoptosis might be partially related to the generation of ROS. Oxidative stress in response to various external stimuli has been implicated in the induction of apoptosis. Oxygen free radicals induce DNA sequence changes and rearrangements that may trigger apoptotic cell death of neuronal cells. There are some reports that suggest ROS are involved in apoptosis related to local anaesthetics. The data presented in this study are generally consistent with those of Tan and colleagues,\textsuperscript{22} where the neurotoxicity of tetracaine was shown to be related to mitogen-activated protein kinase (MAPK) pathways. Interestingly, ROS accumulation may be explained by the fact that higher levels of glutathione and glutathione S-transferase, known to defend against oxidative stress, are expressed in cortical astrocytes, at least in vitro.\textsuperscript{23} In retinal cells, astrocytes protect neurones from oxidative stress in the same way as free radical scavengers do.\textsuperscript{24} Accordingly, this may suggest that tetracaine cytotoxicity may be exaggerated, if astrocytes’ function as free radical scavenger is perturbed.

Our results were obtained through \textit{in vitro} experiments with primary cortical astrocytes cultures, and so it is inappropriate to extrapolate our \textit{in vitro} data to \textit{in vivo} animal experiments or clinical practice. However, since the threshold of pre-seizure activity in tetracaine in humans is 2.5 mg kg\textsuperscript{-1}\textsuperscript{25} and assuming when average body weight of 70 kg, the astrocyte apoptosis in our study occurred below the threshold of pre-seizure activity. This has clinical implications such that excessive treatment with tetracaine could bring out late fatal neurological complications as a result of astrocyte dysfunction.

In conclusion, among commonly used local anaesthetics, tetracaine induced apoptotic cell death in cortical astrocytes, which may be related to oxidative stress. These results suggest that tetracaine has a cytotoxic effect on astrocytes which may contribute to neurological complications after tetracaine administration.
Fig 6 ROS production and cell death in primary cultured cells exposed to tetracaine astrocyte apoptosis. The level of intracellular ROS, abruptly increased 10 min after tetracaine 400 μM treatment, and peaked in primary cultured astrocytes at 30 min (A). Cell death, induced by tetracaine 400 μM for 18 h, was inhibited by the co-treatment with NAC or trolox (B). Data from five independent experiments. Median and inter-quartile range are shown. *P<0.05 is statistically different from the control.

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