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Cytotoxicity of Bupivacaine in Odontoblasts

Hyun Jeong Kim*

Department of Dental Anesthesiology and Dental Research Institute, Seoul National University School of Dentistry, Seoul, Korea

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In this study, the cytotoxicity of commonly used local anesthetics was evaluated on odontoblasts which are essential for pulpal homeostasis *in vitro*. Local anesthetics, such as articaine, bupivacaine, levobupivacaine, lidocaine, mepivacaine, prilocaine, and procaine, were tested on the odontoblast cell line, MDPC-23. The concentration-and time-dependent cytotoxic effects of local anesthetics on odontoblasts were measured by MTT assay. Among local anesthetics treated for 18 h, only bupivacaine significantly showed cell death in a concentration-(LC_{50} = 1.2 mM) and time-dependent manner. To confirm cell death induced by bupivacaine, the observation of cell morphology and FACS using Annexin V and propidium iodide (PI) staining were performed. As a result of Annexin V and PI staining, as well as the morphological change, only bupivacaine induced apoptotic cell death on odontoblasts when compared with levobupivacaine and lidocaine. These results suggest that bupivacaine might affect normal pulpal integrity even after uneventful local anesthesia.

Keywords: apoptosis, bupivacaine, cytotoxicity, local anesthetics, odontoblast, pulp

Introduction

Local anesthetics are generally known to be as very safe (Hass, 1998). However, recently, there are increasing numbers of *in vivo* and *in vitro* reports focusing on the cytotoxicity of local anesthetics (Kytta et al., 1986; Komai and Lokuta, 1999; Friederich and Schmitz, 2002; Tan et al., 2002; Kasaba et al., 2003; Zink et al., 2003; Park et al., 2005). Most dental treatments are carried out under local anesthesia, for this reason, cytotoxic effects of local anesthetics on various oral tissues have been crucial to dental practitioners.

The odontoblasts, one of the major cellular components in the pulp, are located at the most outer layer of the pulp and play an important role in keeping pulpal homeostasis, including pulpal healing and dentin bridge formation in the condition of pulp exposure (Costa et al., 2001; Mantellini et al., 2003; de Souza Costa et al., 2003). During local anesthesia, the odontoblasts around the site of injection should be affected by the diffusion of the local anesthetics. Also, the diffusion of local anesthetics is expected to be much more apparent in intraligamental, intrapulpal and intraseptal anesthesia, which are inevitable for efficient pain management of operative and endodontic treatments (Hargreaves and Keiser, 2004), because those procedures are usually carried out under the pressure.

The MDPC-23, spontaneously immortalized and cloned cell line, was derived from 18-19 day CD-1 fetal mouse molar dental papillae (Hanks et al., 1998; Sun et al., 1998). It has been widely adapted to the study of odontoblast functions, cellular differentiation and regulation of gene expression (Botero et al., 2003; Telles et al., 2003). Moreover, it has been widely used for the study of odontoblasts to external physical and chemical agents and mechanisms for reparative dentinogenesis (Kytta et al., 1986; Zink et al., 2003; Park et al., 2005).

Basically, cell death can occur in two general processes: necrosis and apoptosis. In particular, the apoptotic cell death is a programmed cell death, which can be triggered by various physiologic stimuli, and progresses through a pathway quite distinguishable to necrotic cell death (Majno and Joris, 1995). Based on the recent finding that the appearance of phosphatidylserine (PS) on the outer surface of the cell membrane is one of the typical early apoptotic

*Corresponding author: Hyun Jeong Kim, MD, PhD, Department of Dental Anesthesiology and Dental Research Institute, Seoul National University School of Dentistry, 28 Yeongon-dong, Jongno-gu, Seoul 110-768, South Korea, Tel.: +82-2-2072-3042; Fax.: +82-2-766-9427; e-mail: dentane@snu.ac.kr

events (van Engeland et al., 1998), the method using annexin V-FITC, a labeled protein with a high affinity to PS, was developed and used to detect apoptotic changes easily (Friederich and Schmitz, 2002; Janke et al., 2003). Usually, annexin V-FITC is used in conjunction with a vital dye such as propidium iodide (PI) to identify apoptotic cells. This staining pattern can be analyzed by flow cytometry, which allows the examination if a large number of cells (typically 10,000 per measurement).

The purpose of this study was to evaluate and compare the cytotoxicity of commonly used local anesthetics in dentistry, *i.e.*, articaine, bupivacaine, levobupivacaine, lidocaine, mepivacaine, prilocaine and procaine on the odontoblastic cell line, MDPC-23. Also, if the cytotoxicity of local anesthetics was confirmed, the brief mechanism of these cell deaths, apoptosis or necrosis, can be determined by the staining with annexin V and PI.

Materials and methods

Cell culture and local anesthetics preparation

The MDPC-23 cell line was grown in á-modified Eagle's medium (α-MEM; Gibco, Paisley, UK) with 5% heat-inactivated fetal bovine serum (FBS; Gibco) in a humidified 5% CO_2 incubator at 37°C. Experiments were performed with early passages of the cell line at most 10 passages. Specifically, cells were plated at a density of 2×10^4 cells/mL and grown for 3 days, the medium was then changed, and cultured for an additional 2 days. Local anesthetics, such as articaine, bupivacaine, levobupivacaine, lidocaine, mepivacaine, prilocaine and procaine, all of which were purchased from Sigma (St. Louis, MO), were prepared in various concentrations with serum-free media. The other components in a commercial dental cartridge, such as vasoconstrictor and preservative, were excluded.

MTT assay

The cells were seeded into 96-well plates (1.5×10^5 cells/well). For the cytotoxicity assay, after exposure to local anesthetics, α-MEM containing 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to each well of the plates. Following incubation for 2 h at 37°C, the medium was removed and 100 μL of DMSO (Sigma) was added. The absorbance at 570 nm was recorded using a spectrophotometer (Bio-Tek, Winooski, VT). Results were normalized to the control, which was incubated with no local anesthetics.

Annexin assay measured by FACS

Apoptosis was identified and quantified by staining cells with annexin V-FITC (Pharmingen, San Diego, CA) and propidium iodide (PI) (Pharmingen). Cells (1×10^6 cells/mL) were collected, washed with ice-cold PBS and then resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$]. Then, 5 μL of annexin V-FITC and 10 μL of PI were added to these cell suspensions, to be analyzed with FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

Statistical analysis

The cell viability data from the MTT assay were expressed as mean ± SD (% control). A statistical analysis was performed by Student's t-test using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). And also, the dose-response data of bupivacaine was fitted to a Probit sigmoid equation, yielding 50% of lethal dose (LD_{50}) and slopes (SPSS 12.0, SPSS Inc. Chicago, IL).

Results

In this study of the cytotoxic effect of local anesthetics in odontoblasts, MDPC-23 cells was examined using the MTT assay. Eighteen hours after the treatment of local anesthetics, such as articaine, bupivacaine, levobupivacaine, lidocaine, mepivacaine, prilocaine, and procaine, only bupivacaine significantly induced cell death at 1 mM, compared to the control group (Fig. 1). The bupivacaine-induced cell death occurred in a dose-dependent manner (Fig. 2A) and the value of LD_{50} was statistically approximated to be 1200 μM (Fig. 2B). In contrast, levobupivacaine which has similar anesthetic potency with bupivacaine did not show cytotoxicity up to 2 mM in this study (Fig. 2A). After the exposure to bupivacaine (1200 μM), cell viability was also

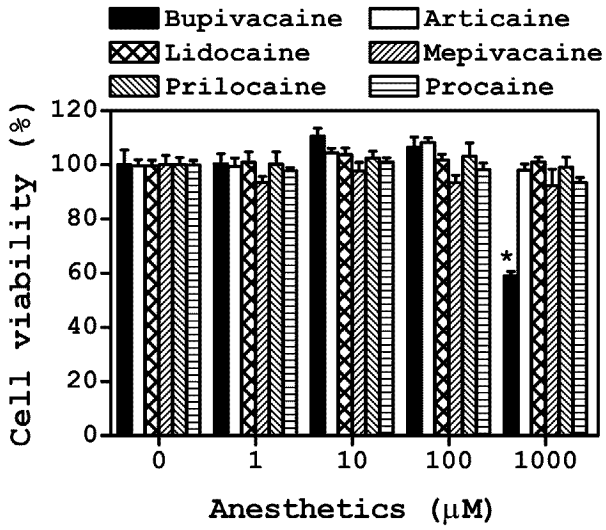

Fig. 1. Cell death induced by local anesthetics in MDPC-23 cells. Cells were treated for 18 h with local anesthetics (0, 1, 10, 100, and, 1000 μM), such as, articaine, bupivacaine, lidocaine, mepivacaine, prilocaine, and, procaine. The percentage of cell viability in MTT assay at each concentration was calculated compared with the untreated control. Data were shown as mean ± SD (bars). *$p < 0.05$ was considered significant.

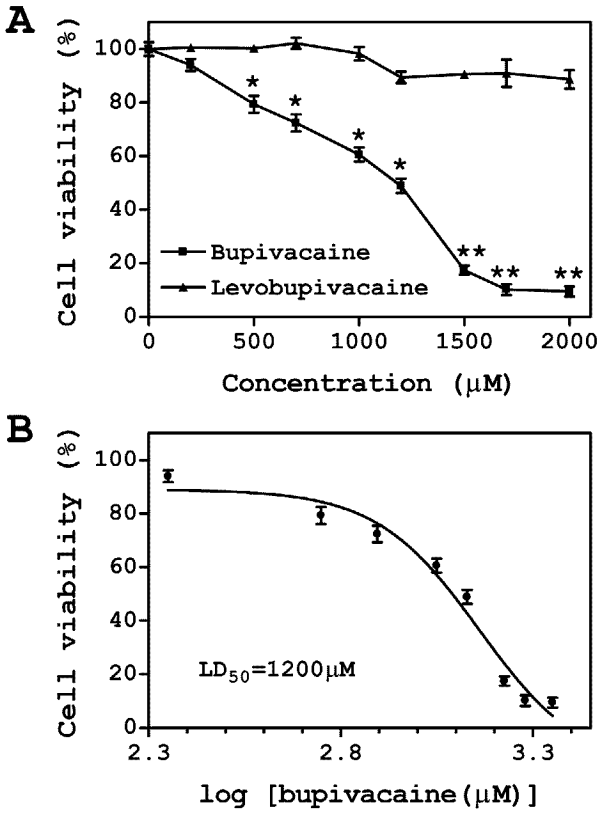

Fig. 2. Bupivacaine induces cell death in a dose-dependent manner. (A) Cells were treated for 18 h with bupivacaine and levobupivacaine (0, 500, 1000, 1500 and 2000 μM). The percentage of cell viability in MTT assay was calculated to be compared with the untreated control. (B) The 50% of lethal dose (LD_{50}) with 95% confidence interval was statistically calculated to be approximately 1200 μM, using GraphPad Prism version 4.00 for Windows. Data were shown as mean ± SD. *p < 0.05 and **p < 0.01 were considered significant.

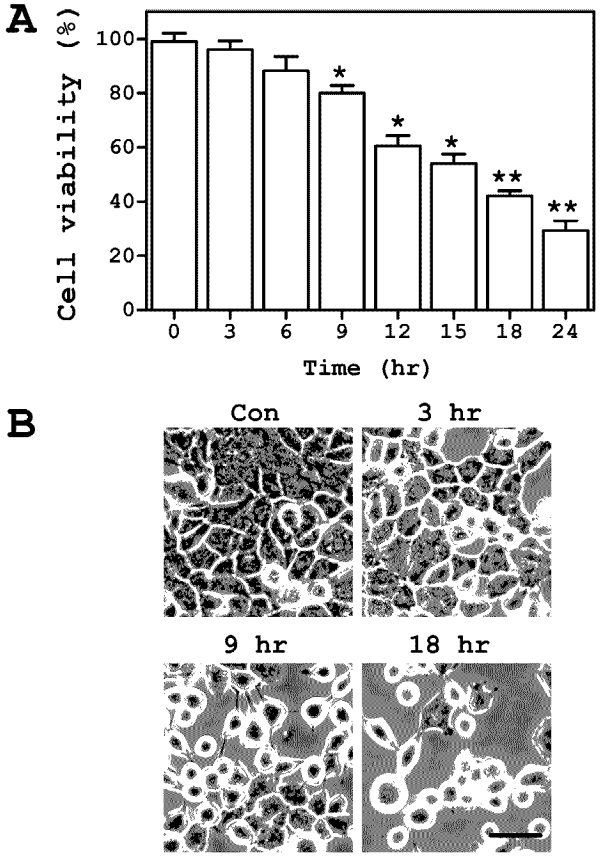

Fig. 3. Bupivacaine causes change of cell morphology in MDPC-23 cells. (A) Cells were treated with bupivacaine (1200 μM) for each time point (0, 3, 6, 9, 12, 15, 18, and 24 h). The percentage of cell viability in MTT assay was calculated to be compared with the untreated control. (B) After the treatment of bupivacaine (1200 μM), the cell morphology was observed under a light microscope (Scale bar = 50 μm). Data were shown as mean ± SD (bars). *p < 0.05 and **p < 0.01 were considered significant.

decreased in a time-dependent manner (Fig. 3A).

To examine whether apoptotic or necrotic cell death was induced by bupivacaine, the observation of cell morphology, staining of Annexin V and PI, and DNA fragmentation were performed. After bupivacaine (1200 μM) treatment, the rounding and shrinkage of cells were observed in a time-dependent manner. Furthermore, odontoblasts lost their cellular integrity and were detached from the bottom of the plate after 18 h (Fig. 3B). In addition to the morphological changes, Annexin V/PI staining was significantly increased in a dose and time-dependent manner using the FACS (Fig. 4). After 18 h incubation with 1200 μM of bupivacaine, 70% of the cells were classified to be apoptotic in FACS (Fig. 4B). Consequently, bupivacaine had also increased the DNA fragmentation in a time dependent manner (Data not shown).

Discussion

In the present study, the cytotoxicity of commonly used local anesthetics was examined on odontoblasts which are essential for pulpal homeostasis, *in vitro*. Since the MDPC-23 cell line is well established and currently used in many studies concerning the pulp tissue (Hanks et al., 1998; Sun et al., 1998), this cell line was selected to use in this study. Local anesthetics, such as articaine, bupivacaine, levobupivacaine, lidocaine, mepivacaine, prilocaine, and procaine, were tested on the odontoblasts. As shown in Fig. 1 and 2, among the local anesthetics treated in this study, only bupivacaine, but not others induced cell death in a dose- and time-dependent manner (LD_{50} = 1.2 mM), while the others did not show cell death.

As a long-acting local anesthetic (the duration of action is approximately 6 – 8 h), bupivacaine has become more popular for prolonged dental procedure and postoperative pain control (Kohase et al., 2002). Interestingly, levobupivacaine, an analogue of bupivacaine with similar anesthetic potency, did not show cytotoxicity examined bupivacaine-induced cell death in this study (Fig. 2A).

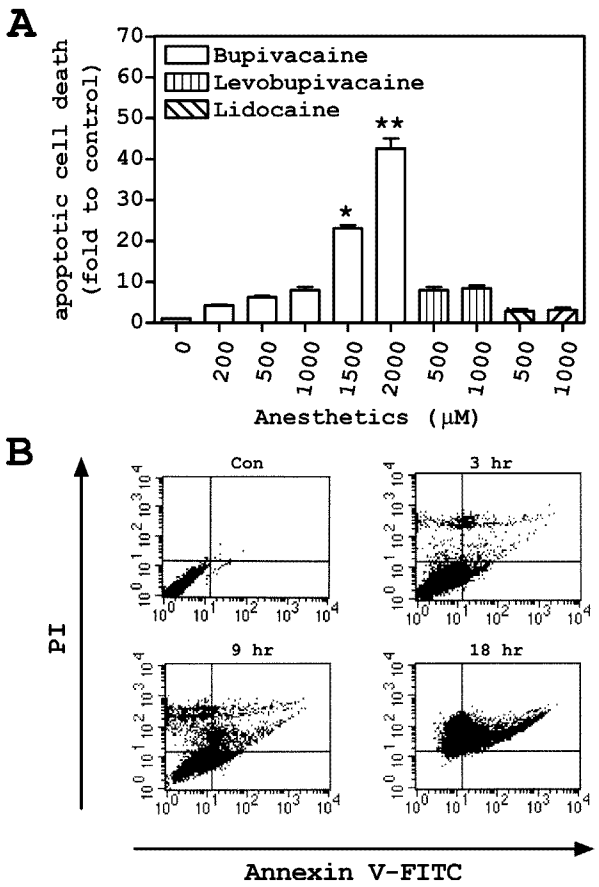

Fig. 4. Apoptotic cell death of MDPC-23 cells treated with bupivacaine. (A) Cells (10,000 cells/ml) were treated with bupivacaine, levobupivacaine, and lidocaine in a dose-dependent manner (0-2000 μM). Apoptotic cells were analyzed by flow cytometry, after being stained with annexin V-FITC alnog with PI. Then, the cells were calculated to be compared with the untreated control (μm to control). Data were shown as mean ± SD. *$p < 0.05$ and **$p < 0.01$ were considered significant. (B) Apoptotic cell death increased in a time-dependent manner after the treatment of bupivacaine (1200 μM).

To define the mechanism of cell death induced by bupivacaine, following methods were performed: the observation of cell morphology, staining of Annexin V and PI, and DNA fragmentation were performed to the high density. The odontoblasts originally exhibit columnar and cuboidal morphology as they are grown to the high density. However, the rounding and shrinkage of the cells became apparent as the time of bupivacaine treatment. And then, odontoblasts lost their cellular integrity and were detached from the bottom of the plate. These results are the typical feature of apoptotic cell death in microscopic findings (Kerr et al., 1972). Bupivacaine also caused the increase in the cell population stained by Annexin V/PI (Fig. 4) and the fragmentation of genomic DNA (Data not shown), the markers of apoptotic cell death. Apoptosis is an active and physiological process for cellular integrity in development and survival. However, it can be also triggered by non-physiologic stimulations and can lead to pathologic conditions (Hall, 1999). Taken together, these results suggest that bupivacaine causes apoptotic cell death in odontoblasts. Furthermore, in dental clinical application, bupivacaine, one of the common medications, could be utilized as an apoptotic trigger in odontoblasts. In addition, the mechanisms of bupivacaine-induced apoptosis should be further investigated in odontoblasts.

These results were obtained through *in vitro* experiments with an immortalized cell line and theoretical stimulation, therefore, it is not possible to simply extrapolate our *ex vivo* data to *in vivo* animal experiments or human research. However, considering that commonly used concentration of bupivacaine for dental procedure is 0.5% (approximately 14.6 mM), bupivacaine may cause the direct damage of odontoblasts at a clinical concentration. Moreover, when bupivacaine is injected with pressure, this probability could be even higher. In addition to our results, there are some clinical reports concerning the cytotoxic effects of bupivacaine (Kytta et al., 1986; Zink et al., 2003).

Several *in vitro* studies have shown the potential cytotoxic effects of bupivacaine on various cells, such as fibroblasts, myocytes, cardiomyocytes, neuronal cells, and Schwann cells (Komai and Lokuta, 1999; Friederich and Schmitz, 2002; Tan et al., 2002; Kasaba et al., Park et al., 2005). In dentistry, however, there have been few reports focusing on the cytotoxicity of local anesthetics in oral tissues. After intraligamental injection of 2% lidocaine, little or no electron microscopic changes in human pulps occurred (Torabinejad et al., 1993). Prilocaine is proved to have the potential to be more toxic than either mepivacaine or lidocaine when applied to gingival fibroblasts of rats (Johnson and Dowse, 1986). To the best of our knowledge, it is the first report about cytotoxicity of local anesthetics on odontoblasts.

In summary, the use of bupivacaine may cause an apoptotic cell death in odontoblasts which are essential for pulpal homeostasis, therefore bupivacaine might affect normal pulpal integrity even after uneventful local anesthesia.

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