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Ropivacaine- and bupivacaine-induced death of rabbit annulus fibrosus cells *in vitro*: involvement of the mitochondrial apoptotic pathway

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SUMMARY

Objective: The purposes of this study were to assess whether local anesthetics (LAs), such as ropivacaine and bupivacaine, could induce apoptosis of rabbit annulus fibrosus (AF) cells *in vitro* and further to explore the possible underlying mechanism.
Methods: Rabbit AF cells at second passage were treated with saline solution and various concentrations of LAs. Apoptosis of AF cells were examined by cell counting kit-8 (CCK-8), Annexin V assays, Hoechst 33342 staining, and Caspase-3, -9 activity assays. The expression of apoptosis-related markers was detected by real-time PCR (RT-PCR) and Western Blot. The JC-1 staining was used to evaluate the change of mitochondrial membrane potential (MMP). Moreover, the levels of reactive oxygen species (ROS) were determined with fluorescent probe DCFH-DA.
Results: The results of flow cytometry indicated that LAs could induce apoptosis of rabbit AF cells in a dose-dependent manner. Apoptosis was confirmed by cell morphology, condensed nuclei and activation of Caspase-3 and -9. In addition, the molecular data showed that LAs could significantly up-regulate the expression of Bax, accompanied by a significant down-regulation of Bcl-2 expression. Furthermore, we also observed that LAs resulted in alteration of MMP and accumulation of intracellular ROS in AF cells. Blockade of ROS production by N-acetyl-L-cysteine (NAC) inhibited LAs-induced apoptosis.
Conclusions: These findings suggest that LAs in clinically relevant concentrations could induce apoptosis of rabbit AF cells *in vitro*, and the mitochondrial pathway was, at least in part, involved in the LAs-mediated apoptosis. Further investigations focusing on the potential cytotoxicity of LAs on IVD cells are needed.

Introduction

In recent years, the therapeutic interventional techniques have been commonly performed to improve the quality of life of patients with low back pain[1–4]. Because of its advantages of minimal invasion and simplicity, the utilization of interventional techniques as a tool for diagnosing and treating spinal pain has sharply increased in the medicare population[5,6]. Local anesthetics (LAs), such as ropivacaine and bupivacaine, are widely used for interventional spinal procedures and generally accepted as being safe. Recently, several studies have focused on the potential toxic effects of bupivacaine on intervertebral disc (IVD) cells[7–10]. Our previous study also showed that LAs decrease the viability of rabbit IVD cells in a

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dose- and time-dependent manner[11]. However, the underlying mechanism of how LAs induce IVD cell death has not been fully elucidated.

Prior evidence suggests that the mechanism by which LAs induce cytotoxicity to IVD cells is predominantly related with cell necrosis rather than cell apoptosis[7,11]. However, these studies measuring toxicity to IVD cells were limited to the immediate or short-term effects of LAs, and the long-term effects of LAs on IVD cells have not been well explored. Interestingly, recent reports concerning LAs toxicity on chondrocytes have suggested the induction of apoptosis is increased significantly at 120 h or 1 week after withdrawal of LAs[12–14], indicating that there exist different mechanisms between the immediate or short-term effects and the long-term effects involved in LAs-induced cytotoxicity. Because IVD cells share a similar phenotype to cartilage chondrocytes, it is reasonable and prudent to examine the long-term effects of LAs on IVD cells and further test whether apoptosis is involved in LAs-induced cytotoxicity.

Apoptosis plays essential roles in the elimination of unwanted, damaged, or infected cells in multicellular organisms. Two main apoptotic signaling pathways have been described: the intrinsic and the extrinsic. The extrinsic pathway is activated by death receptors, leading to the formation of the death-inducing signal complex. This event then results in activation of Caspase-8 to cleavage of Caspase-3 and induction of cell apoptosis. The intrinsic or mitochondrial pathway is activated by suppression of Bcl-2, which gives rise to the permeabilization of the mitochondrial membrane and subsequent the release of apoptotic signaling molecules, for example, cytochrome c[15]. In the cytosol, cytochrome c results in the formation of apoptosome containing cytochrome c, apoptotic protease-activating factor (Apaf-1) and Caspase-9, which then activates Caspase-3 and triggers subsequent apoptosis[16].

In the current study, we first investigated, using different methods, whether LAs induce apoptosis of rabbit annulus fibrosus (AF) cells after withdrawal of LAs. In addition, we assessed the role of mitochondrial pathway in the process of apoptosis induced by LAs through detecting the apoptosis-related markers and the mitochondrial function.

Methods

Isolation and culture of primary AF cells

All experiments in the present study were approved by the Animal Care and Ethics Committee of Huazhong University of Science and Technology and were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Primary AF cell cultures were prepared from the surrounding AF tissue of 3-month-old Japanese white rabbits as described previously[11,17]. When the cells at the second passage reached an 80–90% confluence, they were then seeded onto appropriate culture plates for the following experiments. The cell densities were 3.1×10^4 cells/cm^2 for cell counting kit-8 (CCK-8), flow cytometry, fluorescence microscope and real-time PCR (RT-PCR), and 4×10^4 cells/cm^2 for Western Blot and Caspases activity assay, respectively. The cells were cultured for 24 h leading to adherence prior to experimental treatment.

Treatment groups

We treated rabbit AF cells with the following concentrations of LAs: 0.125%, 0.25% and 0.5% ropivacaine (Ropivacaine HCl; AstraZeneca AB, Sweden); 0.125%, 0.25% and 0.5% bupivacaine (Bupivacaine HCl; Zhaohui Pharm, China). The concentrations used were those of the commercial LAs solution, and their two-fold serially diluted solutions with normal saline. Control groups were exposed to sterile 0.9% saline under the same conditions. After 1-h exposure, cells were immediately re-incubated in fresh culture medium to allow time for recovery for 24 and 120 h.

Measurement of cell proliferation

Cell proliferation as well as the activity of mitochondrial dehydrogenases was measured by CCK-8 (Dojindo, Japan) as described previously[11,18]. After exposure to LAs as described above, 100 μL Dulbecco's modified Eagle's medium/ham's F-12 (DMEM/F-12; Gibco, Grand Island, NY, USA) containing 10 μL CCK-8 solutions were added to each well of the plates. After incubation at 37°C for 4 h, the absorbance at 450 nm was determined with a spectrophotometer (BioTek, Winooski, VT, USA).

The impact of LAs on AF cell proliferation was also tested by the 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay. The assays were performed as recommended by the manufacturer of EdU detection kits (Ribobio, China). Fluorescent images were obtained by a fluorescence microscope (IX71, Olympus, Japan).

Detection of apoptosis by flow cytometry

The AF cells from each treatment group were labeled using Annexin V/propidium iodide (PI) (KeyGen Biotech, China) double staining as described previously[11,18]. All samples were applied to flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest analysis software (BD, USA).

Identification of apoptotic cells by morphology

To certificate the appearance of apoptosis, Hoechst 33342 staining (Beyotime, China) was carried out to observe the condensed and fragmented nuclei. At designed time points, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS). The Hoechst 33342 dye at a concentration of 0.1 μg/mL was added to each well of the plates, and the cells were incubated for 20 min in the dark. Morphologic changes in apoptotic nuclei were observed and photographed using a fluorescence microscope with UV excitation at 350 nm. The quantitative data were expressed as the percentage of apoptotic nuclei relative to total number of nuclei counted.

Detection of Bax, Bcl-2, Caspase-3 and -9 mRNA by RT-PCR

Total RNA was extracted from AF cells of each sample using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and underwent reverse transcription to cDNA with reverse transcriptase (ToYobo, Japan). Specific primer pairs for Bax, Bcl-2 and Caspase-3 were designed previously[18]. The sequences for β-actin and Caspase-9 were as follows: β-actin, forward: 5′-CGAGATCGTGCGGGACAT-3′ and reverse: 5′-CAGGAAGGAGGGCTGGAAC-3′, Caspase-9, forward: 5′-GGAGGATTCCGTGATGTCTGT-3′ and reverse: 5′-CACGTTGTTGATGATGAGGC-3′. Transcription levels of indicated genes and β-actin were detected by RT-PCR on a SLAN Real-Time PCR System (Shanghai Hongshi Medical Technology Co., China) with SYBR PCR master mix (ToYobo, Japan). The relative amount for each gene expression was calculated by the method of $2^{-\Delta\Delta CT}$ using β-actin RNA as an internal control.

Western blot analysis

Total protein was extracted using a Western and IP Cell Lysis Kit (Beyotime, China). To determine the release of cytochrome c from