

## Effects of Local Anesthetics on Cellular Necrosis, Apoptosis and Inflammatory Modulation: Short Review

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### Abstract

**Objectives:** Local anesthetics (LAs) cause the loss of sensitivity to the nociceptive stimulus by the reversible blockade of the neural electrical impulse. These drugs are widely used for pain control in many clinical procedures and in other therapeutic applications. Besides the effects on voltage-gated sodium channels, LAs can modulate different cellular pathways. The review seeks to address the effects of local anesthetics on the cellular viability, apoptosis induction and inflammatory response.

**Methods:** Data was collected from MEDLINE®, Scopus® and Web of Science, the searches were performed between November 2017 and April 2018.

**Results:** LAs induce necrosis and apoptosis in a time and dose-dependent manner. Apoptosis occurs primarily by the intrinsic pathway through the loss of mitochondrial membrane potential. These molecules were able to reduce the release of pro-inflammatory cytokines, chemokines, COX-2, PGE<sub>2</sub> and decrease the activation of NF-κB.

**Conclusion:** The cytotoxic effects of LA are related to high concentrations and prolonged exposure times. LAs activate the intrinsic pathway of apoptosis through cytochrome c release. In concentrations lower than required to perform sensory and motor block, LAs show anti-inflammatory effects.

**Keywords:** Local anesthetics; Necrosis; Apoptosis; Anti-inflammatory response

### Introduction

Local anesthetics (LAs) are effective agents for pain control. They have a well-established role for local and regional anesthesia in both dental and medical practices [1,2]. Despite the variable properties of the several LA molecules, usually the injection of a LA is effective and uneventful, enabling their use in many techniques such as topical anesthesia, infiltration and regional blocks [3].

The structure of the majority of LAs includes hydrophobic and hydrophilic domains linked by an amide or ester-group. The hydrophobic domain is typically represented by an aromatic ring and the hydrophilic portion by a tertiary amine [4]. LA structure and the physicochemical features of each molecule determine the drug potency, onset of action, duration of sensory block and toxicity [5].

The amphiphilic nature of LAs allows their distribution across the cellular membranes [6]. Recently, other effects associated to LA molecules have been explored. Even in lower concentrations than those required to block sodium channels, LAs can modulate several cellular pathways.

LAs induce metabolic changes involving the sarcoplasmic reticulum, mitochondria physiology and reactive oxygen species (ROS) overproduction, which could lead to necrosis and apoptosis [7]. The

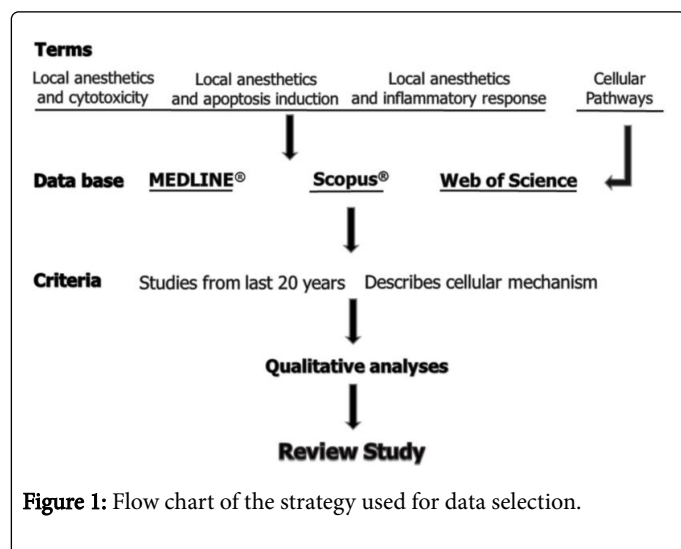
literature has reported cell viability loss in different cell types after exposure to LAs [8]. Moreover, LAs can modulate different cellular pathways in many different cell types, interfering with Ca<sup>2+</sup> homeostasis, inhibition of voltage-dependent calcium channels and mitogen-activated protein kinase (MAPKs) phosphorylation, affecting the wound healing and inflammatory response [9,10].

Several studies have explored other features of local anesthetics besides the blockade of voltage-gated sodium channels. Currently in the literature, there are many data from *in vitro* and *in vivo* studies of the effects of LA on cell viability and inflammatory response. Thus, the present review aims to address the effects of amino-amide and amino-esters local anesthetics on different cell signaling pathways related to necrosis, apoptosis induction and inflammatory response.

### Methods

We aimed to demonstrate how local anesthetics can affect cell viability, apoptosis induction and inflammatory response. Moreover, we explored the main pathways involved. For this, we performed a qualitative analysis on the data from the last 20 years related to the effects of LAs on cell viability, apoptosis and inflammatory response were included. Articles with *in vitro*, *in vivo* and clinical studies that showed some cellular mechanism involved were selected. Articles detailing the cellular pathways were also included.

International indexed data was obtained from MEDLINE®, Scopus® and Web of Science. The searches were performed between November 2017 and April 2018 and only articles in English were selected. The terms used to explore the database were “Local anesthetics and cytotoxicity”, “Local anesthetics and apoptosis induction” and “Local anesthetics and inflammatory response”. Figure 1 shows a flowchart of the strategy used for data selection.



### Local anesthetics cytotoxicity by necrosis induction

The cell lesion induced by chemical stimuli or physical stress is reversible to a certain extent, but if the stimulus is persistent or severe enough, the cell reaches a "point of no return" and suffers irreversible cellular damage, and consequently death. The differentiation between necrosis and apoptosis is based on morphological and biochemical criteria. Necrosis is an energy independent process that cannot be blocked or reversed. It is characterized by the rapid swelling, membrane rupture, cell lysis and DNA degradation. When the cellular damage to the membranes is severe, the lysosomal enzymes are released in the cytoplasm and digest the cell causing the leak of cellular components which inevitably results in necrosis [11].

The exposure of fibroblasts and tenocytes to 0.5% bupivacaine for 30 min, with a subsequent incubation for 1 h and 24 h, led to a significant increase in the necrosis rate [12]. *In vitro*, 15 mM lidocaine induced necrosis in histiocytic lymphoma cells (U937). Direct toxic effects on U937 cells was characterized by rapid disruption of the membrane integrity without DNA fragmentation [13].

Necrosis induced by LAs has been demonstrated in chondrocytes [14]. Hansen et al. [15] reported chondrolysis after surgical treatment in patients submitted to intra-articular injection of bupivacaine for pain control. Interestingly, the patients developed symptoms like pain, stiffness, crepitus and increased pain during movement, which started 3 to 12 months after surgery. Likewise, another study demonstrated significant metabolic changes and reduction in muscle cells viability after bupivacaine intramuscular infusion, with and without epinephrine [16].

Several studies have demonstrated toxicity of LAs to cancer cells in a dose and time-dependent manner [17]. Lidocaine induced cytotoxic effects on breast cancer (BT-20 and MCF-7), colon adenocarcinoma (HT-29 and Caco-2), prostate cancer (PC-3), ovarian cancer (ES-2),

hepatocellular carcinoma (HepG2) and oral squamous carcinoma cells (SCC9 and SCC25), usually in concentrations from 2 mM to 5 mM [18-23]. Apparently, the mechanism of necrosis induction by LAs is unrelated to their role in blocking the Nav [1,16].

### Apoptosis induction by the caspase cascade activation

The apoptosis process is regulated by caspases (a family of 12 different aspartate-specific cysteine proteases), which are synthesized as inactive pro-enzymes. The apoptosis initiation or execution occurs by the caspase cleavage at specific sites [24]. There are two well-characterized mechanisms for caspase-cascade activation: the extrinsic pathway is initiated by death receptors located on cell-surface and the intrinsic pathway is characterized by changes in the mitochondrial integrity [25].

The death-receptors (extrinsic pathway) constitute a family of transmembrane proteins from the tumor necrosis super-family receptors and nerve growth factors [26]. This pathway involves three stages: receptor trimerization induced by a ligand, enrollment of intracellular proteins associated with the receptor and caspases activation [25].

In general, cell-surface death receptors bind specific molecules, such as TNF and CD95L. This linkage forms the death inducing signaling complex (DISC), which is composed by a ligand, the receptor, adapter molecules and the initiator-caspase, especially caspase-8 or caspase-10 in some instances [27]. In the DISC, caspases are activated by dimerization and auto-proteolytic cleavage. This leads to the activation of caspase-3 and the cleavage of other cellular substrates, which are responsible for the morphological changes and cell death [24].

The intrinsic pathway is activated by various stimuli, which include damage to DNA structure, stress on the endoplasmic reticulum, cytokine-deprivation or high affinity binding to antigen receptors. The stimulus alters the permeability of the mitochondria outer membrane, resulting in the release of apoptogenic factors from the internal space of the mitochondrial membrane [25,26].

The outer mitochondrial membrane permeabilization is regulated by a complex interaction of individual members from the B-cell lymphoma protein-2 (Bcl-2) families, which are divided into the pro-apoptotic and the anti-apoptotic. The first comprises Bax/Bak like proteins (Bax, Bak, and Bok) and BH3-only protein groups, while the Bcl-2 anti-apoptotic family members include the Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and Bfl-1/A1 [26].

The apoptosis induction leads BH3-only proteins to activate Bax and Bak-like proteins, promoting a conformational change in the outer membrane of the mitochondria. This alteration results in the creation of pores and cytochrome c release [27]. The cytochrome c released will interact with Apaf-1 and caspase-9, forming the apoptosome. This will be responsible for cleaving the pro-caspase-3 into caspase-3, initiating the cascade of caspases and subsequently apoptosis [22,24].

*In vitro* and *in vivo* studies have demonstrated the induction of apoptosis by local anesthetics on chondrocytes, human neuronal cells, neuronal cells from the dorsal root ganglia in rats and pre-adipocytes [28-30]. At concentrations close to 12 mM lidocaine induced chromatin condensation and internucleosomal DNA fragmentation, that are typical apoptosis features, this effect was dose and time-dependent [16]. Lidocaine and ropivacaine induced apoptosis on human small lung cancer cells (A549 and H520) by disruption of

mitochondrial membrane potential, cytochrome c release and activation of caspase cascade [31].

Werdehausen et al. [24] showed that lidocaine induced the activation of the intrinsic pathway and the blockage of the caspase-8 in the extrinsic pathway did not affect the induction of apoptosis in the human Jurkat T-lymphoma cells. Conversely, the blockade of caspase-9 activation, as well as the over-expression of Bcl-2, reduced the cell apoptosis rate after lidocaine exposure.

Cytotoxic effects of lidocaine and other LAs, which are responsible for inducing apoptosis in several cell types, can be related to mitochondrial disorders. In the protonated form, LAs are mildly acidic amines. They have an acid dissociation constant of about 8, which suggests the possibility of LAs acting as mitochondrial uncouplers, causing the collapse of the protons gradient [32].

Bupivacaine and other LAs can inhibit the “complex I” from the mitochondrial respiratory chain complexes causing an uncoupling of the oxidative phosphorylation, decreased ATP production and the collapse of mitochondrial membrane potential. This sequence results in cytochrome c release that triggers apoptosis, in which the mitochondria could act as the initiator, effector or amplifier of signals [31].

Moreover, studies with bupivacaine and Schwann cells demonstrated that induction of apoptosis could occur by the generation of reactive oxygen species (ROS). ROS affects the depolarization of the internal membrane potential and contributes to the opening of the mitochondrial permeability transition pore [33].

Bupivacaine and procaine in a dose-dependent manner, induced apoptosis in SH-SY5Y cells and mouse DRG neurons. The effects were associated to an increase in ROS production, which caused mitochondrial dysfunction, deregulated mitochondrial  $Ca^{2+}$  signaling and elevated mitochondrial oxidative stress [1,10]. The increase in ROS production by bupivacaine is related to the activation of an AMP-activated protein kinase (AMPK) dependent pathway [34]. Many cellular pathways are regulated by calcium, acting as a second messenger. However, calcium overload is harmful to mitochondrial function [10].

## Modulatory Effects on Inflammatory Response

The inflammatory process is characterized by the activation and recruitment of immune and inflammatory cells towards the injured tissue. This process comprises a complex and well-coordinated series of events that allows an efficient communication between the defense cells, endothelium, epithelium, and smooth muscle. Cytokines play a major role in this process. The binding of these mediators to transmembrane receptors results in changes in the RNA synthesis and protein expression, affecting the dynamics of the extracellular matrix, increasing the fibronectin expression and the permeability of endothelium to the adhesion and infiltration of leukocytes and macrophages [35].

The relationship between cytokines and LAs was previously studied. Lidocaine has shown anti-inflammatory effects in intestinal epithelial cell culture stimulated with TNF- $\alpha$ , inhibiting the secretion of IL-1 $\beta$  and IL-8 as well as increasing the secretion of the antagonist receptor of IL-1 that corresponds to an anti-inflammatory mediator [36]. De Klaver et al. [37] demonstrated the ability of lidocaine to reduce lesions induced by cytokines in endothelial cells and vascular smooth muscle. In another study, a treatment with lidocaine was able to suppress the

release of IL-8 and IP-10 in T-84 and HT-29 intestinal cell strains previously stimulated with TNF- $\alpha$  [38].

De Iulius et al. [39] demonstrated that bupivacaine led to a reduction of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in macrophages, while IL-2 and IL-4 were reduced in rat fibroblasts. Bupivacaine also significantly reduced the PGE<sub>2</sub> production and COX-2 expression in activated mouse macrophages after LPS stimulation [40]. Lidocaine and ropivacaine were able to inhibit the activation of Src by blocking the TNF- $\alpha$  receptor-1 (TNF-R1) signaling. Activation of Src is involved with vascular permeability increase and adhesion of inflammatory cells to the endothelium and it is also involved in tumor metastasis [41].

Lidocaine (2-20  $\mu$ g/mL) suppresses the release of monocyte chemotactic protein 1 (MCP-1), PGE<sub>2</sub>, IL-1 $\beta$  and TNF- $\alpha$  in primary microglia cells activated by LPS [42]. In a similar model, using BV-2 cells (murine microglia) treated with 50 ng/mL endotoxin, the levobupivacaine (25 and 50  $\mu$ g/mL) significantly impairs the upregulation of PGE<sub>2</sub>, chemokine MIP-2 and the cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 [43].

*In vivo*, rats that received lidocaine by intravenous bolus or aerosolized before treatment with LPS had lower levels of IL-1 $\beta$  and TNF- $\alpha$  in bronchoalveolar fluid [44]. Levobupivacaine has been shown to reduce the levels of TNF- $\alpha$  and IL-6 in bronchoalveolar lavage from rats after exposure to 5 mg/Kg of LPS [45].

One of the major signaling transduction pathways is *via* nuclear factor- $\kappa$ B (NF- $\kappa$ B), a transcription factor that uses targeted genes to induce the production of proteins that are able to respond against stress, DNA damage, inflammation, tumorigenesis growth and regulating many vital cell processes [46].

In inflammatory response, NF- $\kappa$ B is a transcription factor in Toll-like receptors signaling pathways, regulating the expression of pro-inflammatory cytokines such as HMGB1, TNF- $\alpha$ , and IL-1 $\beta$  [43]. Among the NF- $\kappa$ B-related proteins, p65 is the most studied, due the interaction with the NF- $\kappa$ B inhibitory protein (I $\kappa$ B) on cellular cytoplasm. The I $\kappa$ B is phosphorylated and degraded when under different physiological or pathological stimuli. The NF- $\kappa$ B is translocated from the cytoplasm to the nucleus, regulating the transcription of the NF- $\kappa$ B target genes [47].

*In vitro*, LA reduced NF- $\kappa$ B activation in dose dependent manner, as shown by Yuan et al. [44] that treated primary microglia cells LPS-activated with lidocaine. Feng et al. [48] showed *in vivo* a reduction in lung lesions caused by LPS after treatment with lidocaine, due to an inhibition of the NF $\kappa$ B activation. Lidocaine inhibits the I $\kappa$ B phosphorylation, avoiding I $\kappa$ B degradation and leading to a reduction in NF- $\kappa$ B nuclear translocation. In addition, it reduces DNA-binding resulting in a down-regulated pro-inflammatory cytokines mRNA expression [41, 44].

Similarly, NF- $\kappa$ B activation induced by LPS in lung tissues and *in vitro* in A549 alveolar epithelial cells can be reduced by levobupivacaine [43]. This LA significantly mitigated endotoxin-induced degradation of I $\kappa$ B, NF- $\kappa$ B nuclear translocation and binding to the microglia DNA [45].

## Conclusion

In addition to the reversible blockade of Na<sup>+</sup> channels and nerve transmission produced by LAs, several effects at the cellular level were identified. We have found *in vitro* and *in vivo* studies showing

cytotoxic effects of LA. Necrosis and pro-apoptotic events caused by LA range from 2 to 15 mM. These concentrations are much higher in comparison to the achieved plasma levels. However, these conditions may occur in some tissues by local injection. Moreover, prolonged exposure times used in some studies exceed the conditions observed in clinical use. Many studies with different cell lines suggest that LAs activate the intrinsic pathway of apoptosis in a dose dependent manner. The protonated form of LAs induces the release of cytochrome c, which is the main pro-apoptotic cellular factor in the intrinsic pathway. However, the activation of the extrinsic pathway of apoptosis has not been demonstrated. In lower concentrations than those required to exert the blockade of voltage-gated sodium channels, LAs exert anti-inflammatory effects. Although the complete mechanism is not well established, LAs have shown anti-inflammatory effects by blocking the release of pro-inflammatory cytokines, such as IL-1 $\beta$ , IL-8, IL-6, and TNF $\alpha$ . The reduction of NF- $\kappa$ B activation is one of the mechanisms involved in the anti-inflammatory activity of LAs. Local anesthetics also induce an inhibition of COX-2 and a consequent reduction in the PGE<sub>2</sub> production. In addition to anesthetic and analgesic properties, antiproliferative and pro-apoptotic effects in cancer cells and anti-inflammatory activity make LAs promising drugs to be explored in other medical fields.

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