Mechanisms of neuro- and cytotoxicity of local anesthetics and their adjuvants
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General introduction and aim of the thesis
General introduction

Local anesthetics were introduced into clinical practice in 1884 by the Viennese ophthalmologist Karl Koller. Since then, local anesthetics have been widely used for many indications, most commonly for local and regional anesthesia. The introduction of high-definition ultrasound at the end of the last century has renewed interest in regional anesthetic procedures, especially peripheral nerve blockades. However, it is well known that all local anesthetics are potentially neurotoxic and cytotoxic, depending on concentration and duration of action. Generally, local and regional anesthesia are considered safe medical procedures. Permanent neurologic damage is rare, but devastating once it occurs. For example, continuous spinal anesthesia and even single shot spinal anesthesia can lead to permanent cauda equina syndromes. This was frequently (1:1300) seen after continuous intrathecal application of lidocaine in high concentrations, a practice that has, in principle, been abandoned. Furthermore, lidocaine is known to induce transient neurological symptoms (TNS) after a single shot intrathecal injection in up to 33% of patients. TNS is defined as segmental radiating pain without concomitant sensory or motor deficit developing within 24 hours after spinal anesthesia. Pain usually subsides within 5 days. The main pathogenic factors of TNS are mechanical stress on nerve roots (i.e. lithotomy position) and local anesthetic neurotoxicity. Although TNS syndrome has been described for all local anesthetics, the incidence is significantly higher when using lidocaine than with most other local anesthetics. For this reason, lidocaine is seen as a prototype for the direct neurotoxicity of local anesthetics.

In animal studies, neurotoxicity of many local anesthetics has been demonstrated, but the mechanism remains incompletely understood. Since blockade of the voltage-dependent sodium channel is the primary mechanism of local anesthetics to block nerve conduction, it has been hypothesized that nerve atrophy may result from long-term application. However, experimental evidence disproved this suggestion. Neither long-term application of blockers of the sodium channel other than local anesthetics, nor long-term inactivation of a sensory nerve led to neurological damage. Therefore, actions other than blockade of the voltage-dependent sodium channel must cause local anesthetic toxicity. It has been demonstrated, that local anesthetics induce elevations of intracellular calcium concentration through external influx or release from intracellular
stores.\textsuperscript{19,20} Furthermore, local anesthetics activate intracellular kinases and inhibit the energy production in the mitochondrion.\textsuperscript{20-24} Apoptosis has been shown to be one mechanism of neurotoxicity in vitro.\textsuperscript{20,25-27} Apoptotic pathways are largely controlled by a family of cysteine proteases called caspases. Different caspases control different pathways of apoptosis. The apoptotic pathways can be divided into two major initiator signaling pathways: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway. They both end in a common executioner pathway initiated by caspase-3. The death receptor is activated by certain ligands extracellularly and combines with the (Fas-associated protein with death domain (FADD) and caspase-8 to form the, death inducing signaling complex (DISC). In the DISC, caspase-8 acts as the initiator caspase of the extrinsic pathway and subsequently activates caspase-3 of the common pathway of apoptosis. In contrast, the intrinsic pathway is initiated at the mitochondrion, which releases cytochrome C and other proapoptotic factors into the cell. The incorporation of pores into the mitochondrial membrane is regulated by the antiapototic protein Bcl-2 and leads to activation of caspase-9 and subsequently to activation of caspase-3 of the common pathway of apoptosis. It has not been determined which pathway is responsible for lidocaine-induced neurotoxicity.

Although lidocaine is the prototype for local anesthetic-induced neurotoxicity, local toxicity has been described clinically and experimentally for almost all clinically used local anesthetics. In vivo studies compared the neurotoxicity of different local anesthetics.\textsuperscript{11-15} To compare all clinically used local anesthetics over a wide dose range is in vivo almost impossible for biometric reasons, i.e. requiring hundreds of experiments. Several in vitro studies demonstrated that local anesthetics induce apoptosis in various tissues.\textsuperscript{20,25,27-29} Unfortunately, most studies just investigated one drug. However, when different drugs were compared, this was not done over a wide dose range, but rather by comparing relatively arbitrary concentrations of the tested drugs. Only Lirk et al. compared the neurotoxicity of lidocaine, bupivacaine and ropivacaine in concentrations equipotent at blocking voltage-gated sodium channels on primary cell cultures of the rat dorsal root ganglia.\textsuperscript{30} They found no difference in toxicity of these three local anesthetics when applied in equipotent concentrations. In this thesis, the toxicity of the eight local anesthetics was compared in neuronal and non-neuronal in vitro models.

A simple method to reduce the toxicity of local anesthetics clinically might be to decrease the concentration by adding different adjuvants.\textsuperscript{31} This is done clinically using a number of different substances in varying concentrations. Some of them are judged as
safe with regard to local toxicity, but for ketamine and midazolam there is considerable doubt about their safety when administered neuraxially.

Ketamine, a uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, is administered epidurally and intrathecally together with local anesthetics for the treatment of pain. However, there is considerable evidence that ketamine and other NMDA receptor antagonists induce neurotoxicity when applied intrathecally over days and weeks. After intrathecal administration, ketamine may damage gray and white matter of the spinal cord, in the subpial region, and around the central canal. Histopathologically, chromatolysis has been described after long-term application of ketamine to the spinal cord. Chromatolysis is a late sign of apoptosis, but not pathognomonic. Thus, there is considerable evidence that ketamine is neurotoxic, but the mechanism of action is unknown. Since ketamine is still used as an adjuvant to different neuraxial blocks, the clarification of the mechanism of toxicity would be interesting in order to judge whether it might further increase the toxicity of local anesthetics. Furthermore, it would be interesting to know whether the newer stereoisomer S-ketamine currently clinically available might be less neurotoxic, as suggested by its reduced centralnervous side effect profile in comparison to the racemate. In addition, ketamine and S-ketamine come in preservative free vials, but also in multi-use vials containing the preservative benzethonium. The preservative benzethonium has been shown to induce apoptosis in several tissues. Because of this property it has even been advocated as a chemotherapeutic agent. Thus, considerable concerns exist when combining the cytotoxic substance ketamine and benzethonium for neuraxial blockades. However, their combined toxicity has never been investigated.

Like ketamine, midazolam, a gamma-amino butyric acid A (GABA$_A$) receptor agonist, is added to local anesthetics during neuraxial blockade in patients. Clinically, it has been demonstrated to prolong analgesia, ensure hemodynamic stability, and reduce postoperative nausea and vomiting. Although, some clinical studies suggest safety, there is considerable concern about the potential neurotoxicity of midazolam. Similar to ketamine, midazolam has received increasing attention for its apoptosis-inducing action in the brain of neonatal animal, when applied intravenously over a long time period. But this effect on the developing brain at its greatest growth spurt and at a time of massive physiologic apoptosis is obviously different from its local effects on differentiated neurons. The basis of this apoptosis induction during development has been proven to be at the GABA$_A$ receptor site. Whether this mechanism is also the basis for the local
neurotoxicity of midazolam is unknown, and may be elucidated with the concomitant application of a receptor antagonist like flumazenil.

After answering the questions regarding the neurotoxicity and its mechanism of ketamine, midazolam and benzethonium per se, we investigated combined toxicities of different adjuvants in combination with lidocaine. Apart from ketamine and midazolam the following substances are most often used clinically: opioids, clonidine, neostigmine, or adrenaline. In vivo or in vitro combined toxicities may lead to subadditive, additive or supraadditive toxicity. Even though opioids and clonidine are known to be non-neurotoxic, they may still increase or decrease the neurotoxicity elicited by lidocaine. We sought to screen a number of additives combined with lidocaine in mixture ratios as used clinically.

**Aims of this thesis**

The first aim of this thesis was to delineate the mechanism of toxicity and induction of apoptosis for the prototype agent, lidocaine. Therefore, we investigated lidocaine, as the prototype of amide local anesthetics, in cell lines with genetically engineered pathways of apoptosis (chapter 2). Lidocaine toxicity was tested in non-neuronal and neuronal cell lines with and without caspase inhibitors in order to identify the concentrations of lidocaine needed to induce apoptosis and necrosis. Furthermore, we could elucidate whether local anesthetics induce apoptosis via the mitochondrial or death receptor pathway.

Clinically, a variety of local anesthetics are used. Although neurotoxicity has been described with almost all local anesthetics, the most relevant symptoms (cauda equine syndrome and TNS following spinal anesthesia), were primarily observed after use of high-dose lidocaine. Consequently, we investigated in our model of neurotoxicity whether some local anesthetics are more toxic than others (chapter 3). We hypothesized that chemical type of local anesthetic (amide vs. ester) or other physicochemical properties might influence toxicity. Furthermore, this comparison of different local anesthetic was also done in a non-neuronal cell line (chapter 4). Stereoisomers of local anesthetics have been introduced into clinical practice in the hope of reducing their systemic toxicity, and therefore we studied whether these L-stereoisomers are also less cytotoxic.

Another way to achieve a less toxic concentration clinically would be to use an additive. This could theoretically reduce the toxicity of the local anesthetic, but only if the additive is not neurotoxic and/or does not increase the neurotoxicity of the local
anesthetic. Two clinically used additives (ketamine and midazolam) have been described as neurotoxic in the past (chapters 5&7). Therefore, we investigated their toxicity in different cell lines as well as primary neuronal cell cultures. If those substances turned out to induce apoptosis via the same pathway as local anesthetics, then additive or possibly supraadditive toxicity might be assumed. Furthermore, we were interested whether the clinically advantageous stereoisomer of ketamine, s-ketamine, would also display reduced neurotoxicity. Some preparations of ketamine contain benzethonium, which has also been shown to induce apoptosis. Therefore, the combined toxicity of benzethonium and ketamine was investigated by means of isobologramms (chapter 6).

Apart from the neurotoxicity induced by ketamine and midazolam alone, the toxicity of mixtures of clinically used local anesthetics together with lidocaine was tested (chapter 8). In a first set of experiments, the ratio of the substances was chosen as most often used in clinical practice. Only the substances, which turned out to increase the neurotoxicity of lidocaine, were further investigated by means of isobolographic analysis.

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