Mechanisms of neuro- and cytotoxicity of local anesthetics and their adjuvants
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General discussion
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The presented data demonstrate that local anesthetics induce apoptosis and, with increasing concentrations, necrosis. Apoptosis is induced via the mitochondrial pathway by incorporating Bax-channels into the outer mitochondrial membrane leading to release of cytochrome c and other proapoptotic substances. The toxicity of local anesthetics correlates with their lipophilicity and thus their conduction-blocking potency and is independent of chemical structure (ester vs. amide) or stereoisomerism. Therefore, the toxic effect does not seem to be induced via a specific “receptor” to which local anesthetics bind as a specific ligand. The concentration-response curves are steep, indicating a rather unspecific, possibly purely physicochemical effect.

Likewise, ketamine and midazolam, which are clinically used as adjuvants to local anesthetics, induce apoptosis via the mitochondrial pathway. Furthermore, their local neurotoxicity is independent of NMDA- or GABA\textsubscript{A}-receptor signaling, respectively. The same mechanism of toxicity as local anesthetics suggested that the combined toxicity could at least be additive. In fact, the combined toxicity of ketamine and lidocaine was additive and the combined toxicity of midazolam and lidocaine subadditive. Likewise, benzethonium increased the toxicity of ketamine in an additive manner. Therefore, combining these drugs in clinical practice for local or regional anesthesia must be done with extreme caution, and if these adjuvants are used, a reduction in local anesthetic dose would seem prudent. In contrast, all other clinically used adjuvants (opioids, clonidine, epinephrine, neostigmine) did not enhance the toxicity of lidocaine in vitro. Therefore, combining them with local anesthetics and thus reducing the required local anesthetic concentration seems a useful concept to avoid local anesthetic neurotoxicity. In summary, the mechanism of toxicity of local anesthetics and clinically used adjuvants was elucidated and drug mixtures with increased toxicity identified.

Critique of methods

Starting point of these investigations was the clinical neurotoxicity of different local anesthetics. Clinically, local anesthetic-induced neurotoxicity is a rare, but potentially devastating, complication of regional anesthesia. Thus, investigating this subject
clinically would require screening of an enormous number of patients. Assuming an incidence of 0.01‰ permanent nerve injury as has been described in observational studies, a group size of 204,916 patients would be required in order to detect a 30% difference in nerve lesion percentage with a 90% power and an alpha of 0.05 (nQuery Advisor 7.0; Statistical Solutions Ltd., Cork, Ireland). Such research will be unfeasible in the light of the low rate of serious neurotoxicity. Furthermore, clinically observed nerve damage is always difficult to diagnose as local anesthetic-induced neurotoxicity. A myriad of other causes might cause perioperative neuropathy.

- Damage to the neural structures by the needles used to perform a nerve block.
- High pressure during local anesthetic injection leading to pressure lesion and ischemia of the nerve.
- Nerve lesions caused by perioperative positioning.
- Nerve damage caused by use of a tourniquet, especially with high cuff pressures and prolonged ischemia time.
- Direct or indirect nerve damage during the surgical procedure caused with knives, retractors, electrocautery or any other surgical instrument.
- Unrelated nerve infection/inflammation acquired perioperatively causing chronic pain syndromes, numbness or paralysis.
- Neuromuscular pain syndromes provoked by other causes in the perioperative period.
- Preexisting neuropathies (e.g. diabetic neuropathy) may increase the susceptibility of nerves to pressure or toxicological injuries.

Thus, it is nearly impossible to evaluate toxicity of local anesthetics and their adjuvants using clinical investigations. Furthermore, clinical trials are inadequate to discern mechanistic pathways. Therefore, we chose an experimental setting to investigate the subject. We studied the subcellular mechanism of apoptosis induction by local anesthetics and their adjuvants in an in vitro model. Proof of in vivo neuroapoptosis following application of local anesthetics is, to date, lacking, even though apoptosis was identified in vitro 15 years ago. Of course, in many in vivo studies, local anesthetic-induced neurotoxicity was demonstrated and with all
likelihood the neurotoxicity is induced by neuroapoptosis. However, no study demonstrated apoptosis directly in an in vivo model.

In addition, we wanted to compare various local anesthetics, their stereoisomers and antagonists, and clinically relevant adjuvants (in total 20) over a wide concentration range, and using clinically meaningful combinations of the substances. Therefore, it seemed prudent to use an in vitro model instead of an in vivo model, which obviously might resemble the clinical situation more.

Thus, the in vitro models chosen here are obviously distant from the clinical situation. The cell culture models have several limitations in translating data to the in vivo or clinical situation: Human neuroblastoma and Jurkat T-lymphoma cells are immortalized, growing and dividing during the local anesthetic exposure rather than being mature terminally differentiated neurons. Neuroblastoma cells are derived from malignant neural crest cells, which would ordinarily differentiate into the sympathetic chain, adrenal glands, or dorsal root ganglia. Nevertheless, despite those limitations, our model of a human cell line seems even more sensitive in detecting minor differences between different local anesthetics compared to in vivo studies. Furthermore, we retested our results from those tumor cells in primary rat glial or neuronal cell cultures. Although tumor cells like neuroblastoma or Jurkat-cells often have altered apoptosis pathways disinhibiting their growth, the tumor cells were more sensitive to the investigated substances than the primary neuronal or glial cell cultures or than their toxicities described in vivo.\textsuperscript{6-12}

The concentrations of local anesthetics that induced apoptosis in our model are within the same range as those observed intrathecally after single-shot spinal anesthesia in man or primates and in sciatic nerves of rats after intraneural injection.\textsuperscript{13-15} Therefore, potentially neurotoxic concentrations are reached clinically. However, after a single-shot spinal anesthesia or peripheral nerve block these concentrations are only observed during the first hour after injection, whereas in the presented cell culture model the concentrations were kept constant for 24 h. It is well known that beyond the concentration, the duration of exposure to a local anesthetic is important for the development of neurotoxicity, therefore permanent neurotoxicity after single application is a rare complication clinically. In a small sample of patients undergoing peripheral nerve blockade, intraneural injection of lidocaine 2\% (\approx 78 mM) for single shot sciatic nerve block did not lead to any functional nerve damage,
although this concentration is more than eight times the in vitro LD$_{50}$ concentration observed here.$^{15}$ Again, direct comparison of these results is difficult, since the concentrations observed after a single shot nerve block will decline rapidly while the concentrations were kept constant in our in vitro experiments.

Another important disadvantage of our model is that the cell line and cell cultures were composed just of one cell type. Thus, we could not evaluate effects that might have been due to tissue interactions. The most important point possibly missed is the potentially vasoconstrictive action of tested substances. Especially epinephrine and clonidine are known to have a strong vasoconstrictive action via vascular alpha-adrenoreceptors. This effect can lead to increased tissue concentrations of local anesthetics over a longer period of time. Since local anesthetic toxicity is dependent on both concentration and exposure time, epinephrine and clonidine may by this mechanism increase the neurotoxicity of local anesthetics. Such possible effects cannot be identified in our in vitro models. Furthermore, the local anesthetics themselves and even different stereoisomers have variable vasoactive properties.$^{16-19}$

Obviously, these effects may be missed in our studies. Since the vasoconstrictor action of local anesthetics seems to correlate with lipophilicity the toxicity of lipophilic substance may in vivo be even increased.$^{20}$ However, the local anesthetic concentrations inducing vasoconstriction are in the millimolar range and thus may only be reached for a short period clinically after a single shot block. Since for the studies presented the concentrations were kept constant this vasoconstrictor effect of the local anesthetic could not influence the observed neurotoxicity.

In conclusion, the experimental setting of the presented investigations is not a precise resemblance of the clinical situation during regional anesthesia. Therefore, any conclusions from our experiments to the clinical situation must be drawn with extreme caution. However, the questions and hypothesis investigated and answered in this thesis cannot be handled with in vivo or clinical investigations for the reasons mentioned above. Thus, the most appropriate methods were used to investigate mechanisms of local anesthetic toxicity and to compare toxicities of different local anesthetics, their adjuvants and mixtures of both over a wide range of concentrations.
**Critique of results**

An almost uniform observation during our studies was the switch from apoptosis to necrosis as mechanism of cell death within a narrow concentration range. At first glance this seems surprising, but strong noxious insults are generally more likely to result in necrotic rather than apoptotic cell death. At higher concentrations local anesthetics may trigger necrosis by gross alterations of cellular integrity, e.g. by disturbance in ion fluxes or loss of membrane integrity. Furthermore, since local anesthetics in millimolar concentrations interfere with the mitochondrial energy production, one may speculate that higher concentrations deplete cellular ATP, which is required for apoptosis.

Here it was demonstrated for the first time that lidocaine induces apoptosis via the mitochondrial pathway. Mitochondrial membrane depolarization had been demonstrated before by others, but mitochondrial membrane depolarization occurs also in other circumstances of altered cell viability. Bcl-2-overexpression has been reported to decrease the toxic effects of ropivacaine in human keratinocytes, although in this study apoptosis was only detected by the loss of procaspase-3 using semiquantitative analysis. Thus, published literature supports the finding described here, but the mechanisms of toxicity have never been investigated systematically.

The studies of Johnson and Lirk tried to evaluate the mechanisms of local anesthetic induced apoptosis employing different pharmacological enzyme inhibitors. However, the pharmacological inhibition of enzymes is much more fallible to error, because the specificity of an inhibitor must always be questioned. Therefore, a model with genetically engineered apoptosis pathways was used which is much more specific than pharmacological inhibitors.

Some studies have attributed the cytotoxicity of local anesthetics to an unspecific membrane effect of a detergent. However, our finding that distinct alterations of apoptotic proteins decrease apoptosis disproves that the detergent-like effect of lidocaine is the principal cause of apoptosis induction. Nevertheless, it is conceivable that higher concentrations of local anesthetics inducing necrosis might be caused by a detergent effect of local anesthetics on the cell membrane.

As mentioned in chapter 1, when we started comparing neurotoxicity of different local anesthetics, just a few papers comparing various local anesthetics existed, and no study comparing them over a wide range of concentrations. Lirk et al. demonstrated that ropivacaine, lidocaine and bupivacaine in equipotent...
concentrations were equally toxic. However, they did not investigate each local anesthetic over a wide concentration range. Almost parallel with our paper, two other studies were published on that subject with similar methods. Perez-Castro and co-workers compared the cytotoxic effects of short-time (10 min) exposure with procaine, mepivacaine, lidocaine, chloroprocaine, ropivacaine and bupivacaine in human SH-SY5Y neuroblastoma cells and found the same order of toxicity as seen in the data presented here. Since local anesthetic induced neurotoxicity is known to be concentration- and time-dependent, it is not surprising that higher concentrations for a shorter duration of exposure induces apoptosis. In contrast to the observations of Perez-Castro and our group, Lee et al. could only observe apoptosis induction by tetracaine in primary rat cortical astrocytes by means of Hoechst 33258 staining, PARP and procaspase-3 immunoblotting. However, they investigated only concentrations below 1 mM and thus only found cell death and apoptosis induced by tetracaine. This result is compatible with those of Perez-Castro and ours. However, the conclusion Lee et al. draw is elusive due to methodological problems. They compared equimolar rather than equipotent concentrations of the local anesthetics or, what would have been even better, the whole toxicity range. Nevertheless, their results also reconfirm our investigation.

There are few in vivo studies comparing different local anesthetics for biometric reasons. Kalichman et al. found a good correlation between the potency of four local anesthetics and their neurotoxicity by means of semi-quantitative examination of the sciatic nerve in rats. Sakura et al. compared in a rodent model of continuous spinal anesthesia equipotent concentrations of lidocaine and bupivacaine with an equal sensory deficit after 4 days. However, Yamashita et al. found bupivacaine and ropivacaine to be significantly less toxic then lidocaine and tetracaine.

Ketamine and midazolam have been advocated as local anesthetic adjuvants for regional anesthesia in men, since they seem to enhance duration and quality of analgesia. Although, there are studies without serious neurological complications in hundreds of patients, there is still considerable doubt about the neurotoxic potential of ketamine and midazolam. Vranken et al. demonstrated in rabbits and patients morphologically neurotoxicity of ketamine with unaltered neurological function. Similarly, in various animal models of regional anesthesia, midazolam
induced neurotoxicity.\textsuperscript{42,43} However, the mechanism of neurotoxicity of these substances has never been investigated.

Ketamine and midazolam as other NMDA-receptor antagonist and GABA-receptor agonist used intravenously for general anesthesia have shown to induce neuronal apoptosis in rodent and primate neonatal brain.\textsuperscript{44-46} This neuroapoptosis seems to be induced via the mitochondrial and the death receptor pathway.\textsuperscript{47} Worth mentioning, using these substances in higher concentrations for neuraxial anesthesia in adult animals is a different situation, because the neurons are differentiated and are exposed to much higher concentrations then when the substances are applied intravenously. Thus, it is not surprising that these substances induced apoptosis via the mitochondrial pathway, thus by another mechanism then in the neonatal brain. Apoptosis induction, independent of NMDA-, GABA\textsubscript{A}- or benzodiazepine-receptor signaling, is therefore also in-line with the different apoptotic pathway. Summarized, the apoptotic mechanisms induced by midazolam and ketamine in our model are different from the neurotoxicity of these substances seen in neonatal models. We showed that the preservative benzethonium further increased the toxicity of ketamine in an additive manner and therefore should be avoided in mixtures used for regional anesthesia.

While investigations regarding local anesthetic-induced neurotoxicity are comparatively abundant (578 pubmed hits; July 2012), the literature on neurotoxicity of adjuvants (or additives) is rather sparse (10 hits in pubmed with just 3 original articles, 2 of them, including our publication, truly related to the subject). Thus, the only other study addressing this subject was done parallel to ours in primary dorsal root ganglia cell cultures of the rat. These authors applied ropivacaine in a toxic concentration for 2 hours on the cells with one or a combination of one of the following adjuvants: clonidine, buprenorphine, dexamethasone or midazolam. In accordance with our results midazolam increased the toxicity of ropivacaine. However, they also observed that the opioid as well as clonidine alone and in combination with ropivacaine showed some neurotoxicity. This may be due to the short exposure time and the high concentrations used (up to 1.000-fold higher than in our model). When they exposed their primary cell cultures to ropivacaine over 24 hours in clinically used concentrations - in accordance with our observations - only midazolam increased the toxicity of ropivacaine. Thus, based on these in vitro data
the use of midazolam as adjuvant to local anesthetics in clinical practice should be
done with extreme caution if not avoided completely.

Future research
All classic local anesthetics are neurotoxic and their neurotoxicity correlates with their
conduction-blocking potency. All local anesthetics are structural analogs, which bind
specifically to the same site of the voltage-dependent sodium channel. Therefore, it is
highly unlikely that newly developed local anesthetics will have a better therapeutic
range or even be devoid of neurotoxicity. In the last two decades two local
anesthetics were introduced into clinical practice, levobupivacaine and ropivacaine.
Both are stereoisomers and thought to have less systemic toxicity than the racemate.
With regards to local neurotoxicity they do not differ as demonstrated in this thesis.
Therefore, future research should not focus on development of even more structural
analogs or slow-release preparations of local anesthetics in order to overcome
neurotoxicity, but on development of completely different blockers of the voltage-
dependent sodium channel. Interesting options are saxitoxine and tetrodotoxine,
naturally occurring toxins with sodium-channel blocking action.\textsuperscript{48-50} They block the
sodium channel at another site than classical local anesthetic and seem completely
devoid of local neurotoxicity. Furthermore, there are structural analogs of these
substances that bind to a specific subtype of sodium-channels.\textsuperscript{51,52} Such a pure
analgesic block via NaV\textsubscript{1.7} sodium channel subtype without blockade of other sensory
or motor fibers would be a great improvement in many areas of pain therapy.
Besides, more specific blockade of pain fibers may be accomplished by using ligands
to other receptors such as TRPV1 (transient receptor potential cation channel,
subfamily V, member 1 also known as vanilloid or capsaicin receptor) or TRPA1
(transient receptor potential cation channel, subfamily A, member 1). These receptors
are almost exclusively located on C-fibers transmitting pain and thus have a selective
action only on pain fibers.\textsuperscript{53,54} However, this research is far away from clinical
practice and it may take decades before such substances will replace classical local
anesthetics in clinical practice.
Meanwhile, one could focus on substances diminishing the neurologic injury of local
anesthetics and some of them have been described including caspase-inhibitors,
kinase inhibitors and radical scavengers.\textsuperscript{7,8,10,55} However, such substances are highly
experimental. Since neurotoxicity of local anesthetics is a very rare (but serious)
problem clinically, the standard addition of substances possibly reducing neurologic injury might be a greater risk than benefit because of their own toxicity and side effects. Since the concentration-dependence of local anesthetic has been demonstrated here and by others, solutions with lower local anesthetic concentration accomplished by the addition of adjuvants are a good and already practiced method to minimize the risk of neurotoxicity. While during epidural anesthesia it is standard to use a mixture of opioid and local anesthetic and possibly adding some other drugs, there are no studies investigating the effects of adjuvants for continuous peripheral nerve block. Therefore, not only the possible neurotoxicity of these mixtures should be investigated more extensively in vitro and in vivo, but also their effectiveness during peripheral nerve blockade deserves a lot more attention especially in clinical studies.

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