Mechanisms of neuro- and cytotoxicity of local anesthetics and their adjuvants
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Chapter 10

Summary
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It was the aim of this thesis to discern the mechanism of cyto- and neurotoxicity of local anesthetics and their adjuvants in various in vitro models of neuronal, glial and mesenchymal cells. The extent and mechanism of lidocaine-, ketamine- and midazolam-induced apoptosis were evaluated in a cell line with genetically modified pathways of apoptosis. Furthermore, the cyto- and neurotoxicity of the local anesthetics most frequently used clinically and their stereoisomers was compared. A number of clinically used adjuvants and preservatives were also analyzed with regard to their mechanisms of cytotoxicity. In a following step, the effect of local anesthetics and adjuvants on their combined neurotoxicity was analysed.

In order to elucidate the mechanism of lidocaine-induced cytotoxicity and apoptosis, lymphoma T-cells (Jurkat) were incubated for 24h with increasing concentrations of lidocaine. The signs of toxicity and apoptosis (cell viability, mitochondrial alteration, cytochrome c release, caspase activation and phosphatidylserine externalisation) were investigated by trypan blue staining, substrate fluoroscopy, JC-1 and 7AAD/PI fluorescence-activated cell-sorting (FACS) analysis, and immunoblotting. Furthermore a pancaspase inhibitor was added in order to quantify caspase-dependent cell death (apoptosis). Lidocaine induced primary apoptosis at low millimolar concentrations (up to 6 mM) and primary or secondary necrosis at higher concentrations. In a second set of experiments, genetically engineered cells with either a defective mitochondrial pathway (Bcl-2 overexpressing or caspase-9 deficient) or defective death receptor pathway (caspase-8 deficient or FADD deficient) were incubated with lidocaine. Only cells with deficient mitochondrial pathway were protected against lidocaine-induced apoptosis. Finally, neuroblastoma cells were incubated with lidocaine with and without a pancaspase inhibitor. Also in
this neuronal cell line lidocaine at low concentrations (<12 mM) induced apoptosis, whereas at higher concentrations it induced necrosis. After elucidating the mechanism of apoptosis of lidocaine, we compared the apoptotic potency of eight clinically used local anesthetics by flow cytometry and correlated these potencies with physicochemical properties and conduction blocking potency in a neuroblastoma cell line. The neurotoxicity of the local anaesthetics correlated with their lipid solubility and thus their conduction blocking potency, but was independent of chemical class (amide or ester), molecular weight, protein binding or pKa. Ketamine is an uncompetitive NMDA receptor antagonist with analgesic properties sometimes used as an adjuvant in regional anesthesia. In our model of genetically modified apoptotic pathways it was demonstrated that ketamine induces apoptosis via the mitochondrial pathway independent of death receptor signaling. Increasing concentrations led to a switch in cell death mechanisms from apoptosis to necrosis. In neuronal cells, marginally less toxicity was observed with the S(+) isomer in comparison to the racemate. Therefore, it is unlikely that the toxicity is induced via the stereosensitive NMDA receptor, since S(+) ketamine has a 3-times stronger binding/action at this receptor. The clinically used preservative benzethonium, when combined with S-ketamine, additively increased the toxicity in lymphoma, neuroblastoma, and primary rat astrocyte cell cultures as revealed by isobolographic analysis. Like ketamine, the GABA\textsubscript{A} agonist midazolam induced apoptosis via the mitochondrial pathway. Again, in neuroblastoma cells and primary rat neurons the mechanism of cell death was concentration-dependent with increasing concentrations changing from apoptosis to necrosis. It could not be inhibited by the competitive GABA\textsubscript{A} antagonist flumazenil, ruling out that the toxicity is induced via the GABA\textsubscript{A} receptor or the peripheral benzodiazepine receptor – a 18 kDa
translocator protein located in the outer mitochondrial membrane known to induce apoptosis when activated.

The adjuvants most frequently used clinically, when added to toxic concentrations of lidocaine, were evaluated in neuroblastoma cells and primary rat astrocytes. Addition of sufentanil, morphine, clonidine, epinephrine and neostigmine to lidocaine did not enhance the neurotoxicity of lidocaine. Ketamine increased the toxicity of lidocaine in an additive manner, while midazolam displayed only subadditive toxicity.

In conclusion, local anesthetics, ketamine and midazolam induce apoptosis via the mitochondrial pathway independent of death receptor signaling. The lipid-solubility and thus the potency of local anesthetics determine their toxicity. Therefore, the therapeutic range of the investigated local anesthetics is about equal and their structure does not influence their cytotoxicity. New substances resembling classical local anesthetics will probably have a similar therapeutic window, and will thus still be neurotoxic. The adjuvants ketamine and midazolam as well as the preservative benzethonium enhanced cytotoxicity. Even though our experimental model is not an accurate representation of the clinical situation, we advise caution in using these substances for local and regional anesthesia. More commonly used, and equally effective, adjuvants such as epinephrine, clonidine and opiates should be used instead. They did not enhance direct lidocaine neurotoxicity and, in the clinical situation, their addition allows for the use of lower concentrations of local anesthetics. Future innovative research may develop other types of voltage-dependent sodium-channel blockers and may focus on more specific blockers aiming at specific voltage-dependent sodium-channel subtypes or other type of voltage-dependent channels transmitting pain.